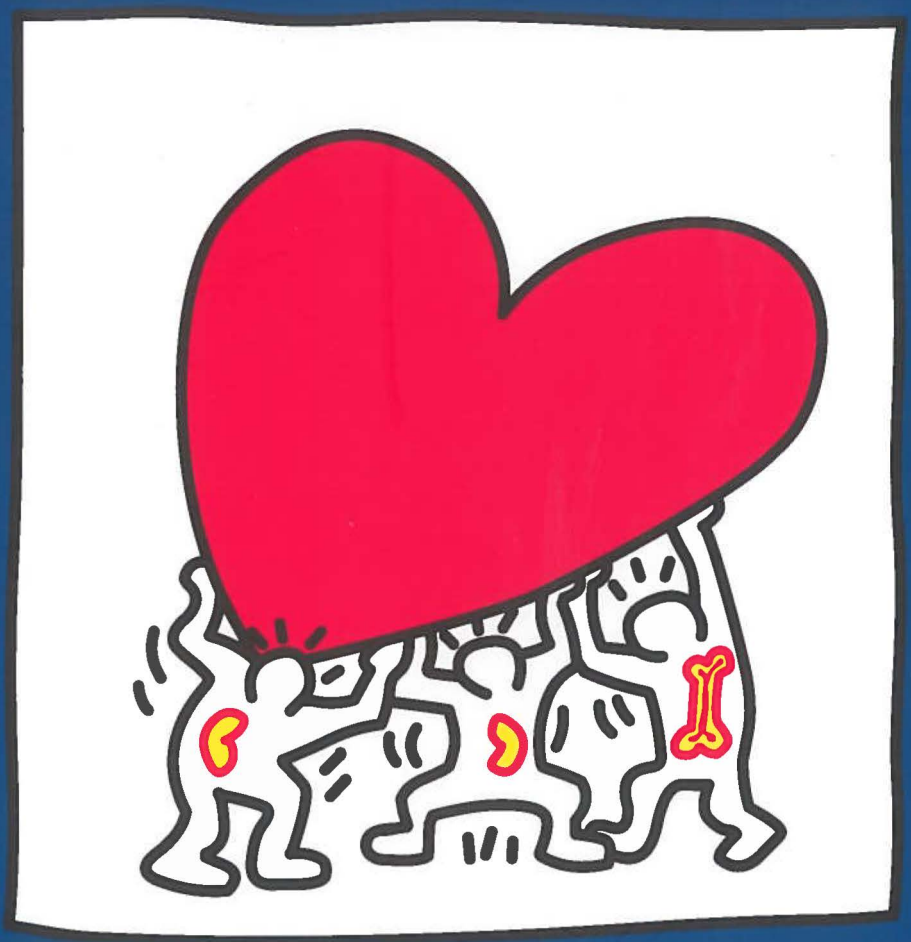

Erythropoietin in Heart Failure
Pathology and Protection

B. Daan Westenbrink

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Stellingen

Behorende bij het proefschrift:

Erythropoietin in Heart Failure
Pathology and Protection

Door B. Daan Westenbrink

1. Hemodilutie draagt bij aan de hoge prevalentie van anemie bij patiënten met hartfalen. *(Dit proefschrift)*

2. Hartfalen leidt tot beenmergdisfunctie. *(Dit proefschrift)*

3. De centrale rol van een verstoorde hemodynamiek in de pathogenese van anemie bij patiënten met hartfalen, suggereert dat hartfunctieverbetering anemie kan tegengaan. *(Dit proefschrift)*

4. Erythropoiëtine verbetert de hortfunctie in ratten met hartfalen. Deze effecten van erythropoiëtine kunnen niet worden toegeschreven aan de verhoging van het hematocriet. *(Dit proefschrift)*

5. Hartfunctieverbetering door erythropoiëtine in ratten met hartfalen wordt voornamelijk bewerkstelligd door stimulatie van de erythropoiëtine receptor in het hart. *(Dit proefschrift)*

6. Stimulatie van de Vasculor Endothelial Growth Factor (VEGF)-productie door ischemische cardiomyocyten is cruciaal voor erythropoiëtine geïnduceerde hartfunctieverbetering. *(Dit proefschrift)*

7. Introductie van fase 0 onderzoek kan een belangrijke reductie van tijd en kosten bewerkstelligen bij de ontwikkeling van nieuwe geneesmiddelen.

8. Wanneer je jezelf schaart onder "believers", wordt wetenschap een religie.

9. De promovendus is geboat bij een Afrikaanse beleving van tijd.

10. Het broeikaseffect lijkt vooral een probleem bij mooi weer.

11. Als de reglementen van de Formule 1 worden toegepast op de Tour de France, zouden de wielrenners alleen nog op driewielers zonder versnellingen mogen rijden.

12. All work and no play makes Jack a dull boy. *(James Howell)*

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Pathology and Protection

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Berend Daan Westenbrink
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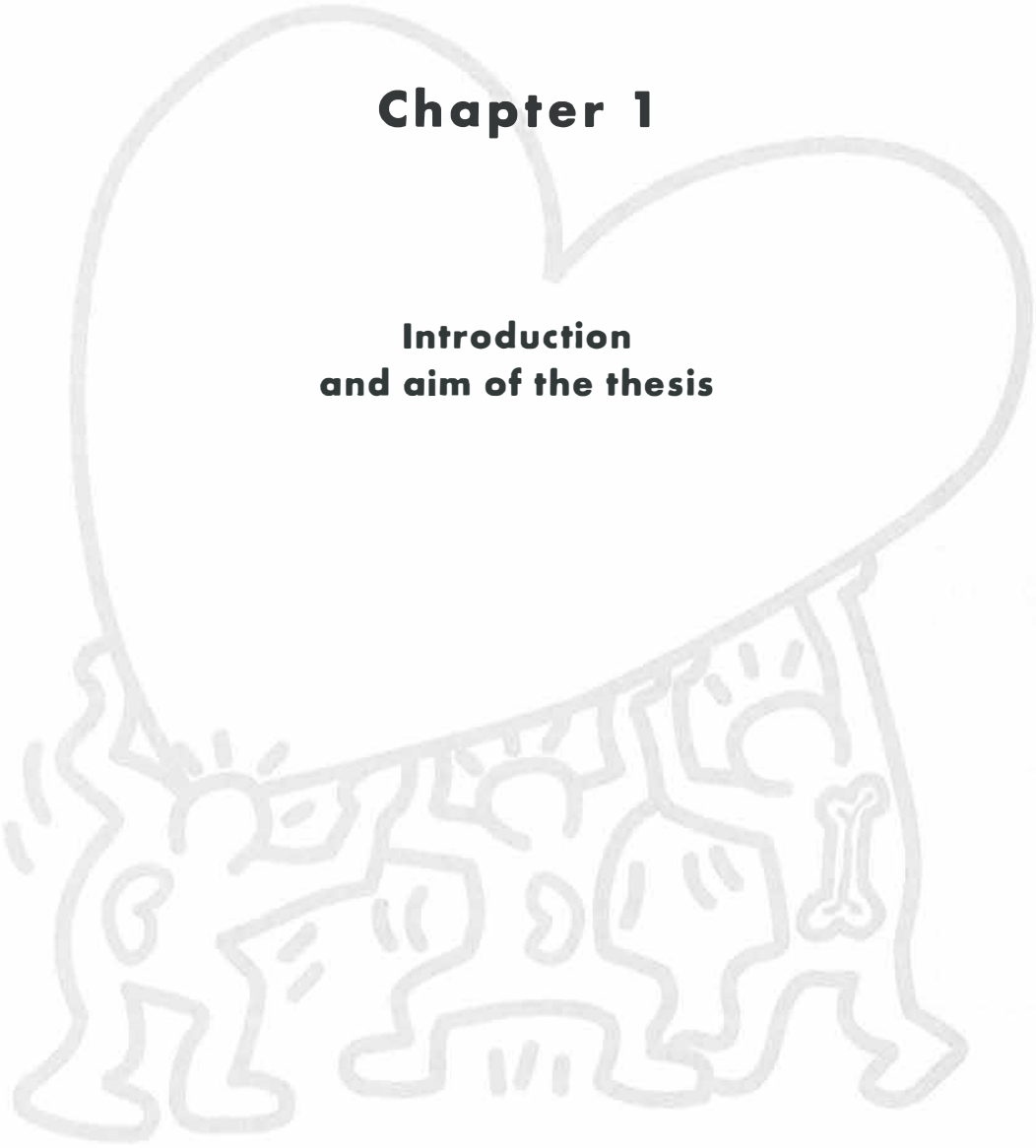
Chapter 1

Introduction
and aim of the thesis



Introduction

Chronic heart failure (CHF) represents a complex of symptoms related to impaired cardiac function which affects 5 million people in the US and 7 million people in Europe.[1] CHF is the final common endpoint of cardiac disease and a leading cause of death in the Western world. Although significant morbidity and mortality benefits have been made over recent years, still over half of CHF patients die within five years.[2] In addition to increased mortality, frequent hospital admissions, extensive diagnostic testing and the use of multiple pharmacological agents account for an immense healthcare expenditure, estimated at $25 billion in the US annually.[1]

The mainstay of treatment of CHF is pharmacological and focused on the suppression of neurohormonal activation in order to attenuate the progression of cardiac dysfunction.[2] Besides cardiac dysfunction, CHF is often accompanied by dysfunctions of other organs, including the endothelium, lungs, kidneys, liver, skeletal muscle and the brain, as well. The extent of dysfunction of other organ functions might have more impact on prognosis than the ventricular dysfunction itself. For example, the extent of kidney dysfunction more accurately predicts outcome in CHF patients than left ventricular ejection fraction (LVEF).[3] Research on the etiology and possible reversal of these collateral organ dysfunctions in CHF might therefore significantly improve clinical outcome.

The importance of anemia in heart failure

Anemia is frequently found in CHF-patients and its presence has been associated with significantly impaired morbidity and mortality.[4, 5] Therefore, anemia might be regarded as an additional collateral organ dysfunction of the CHF-syndrome. Anemia causes chronic volume overload to the left ventricle, which results in increased oxygen consumption, left ventricular dilatation and left ventricular hypertrophy, thereby further reducing cardiac function in CHF. These detrimental effects of anemia in heart failure patients have already been described in Fishbergs' textbook of heart failure in 1937.[6] Despite the longstanding knowledge of the consequences, anemia in CHF it is currently still under recognized and under treated in clinical practice. A recent survey of more than 6000 ambulatory CHF patients showed that although anemia was present in 17% of patients, clinical evaluation of anemia was only performed in 3%.[7] The severe under evaluation is probably attributable to the fact that the etiology of anemia is often unexplained and thus treatment options are limited. Resolving the preponderant etiology is therefore warranted.

Role of erythropoietin in the pathophysiology of anemia

Erythropoietin (EPO) is the principal hormone responsible for the regulation of red blood cell production. EPO is secreted in the kidney in response to hypoxia and in turn targets the bone marrow to increase the production of red blood cells. The high incidence of renal dysfunction in CHF patients suggests that impaired EPO-production by the kidney could be the underlying cause. Moreover, it has been suggested that the sensitivity of the bone marrow to EPO is also impaired. Both dysregulation of EPO synthesis or an altered response to EPO might cause anemia in CHF. EPO is of

particular interest because recombinant human EPO has been used to treat anemia for several decades and preliminary studies have demonstrated efficacy in CHF patients.[8-11] Nevertheless, a central pathophysiological role for endogenous EPO in anemia in CHF remains to be established.

Effect of EPO on the myocardial microvasculature

In heart failure, microvascular adaptation to cardiomyocytes hypertrophy is required to compensate for the increased metabolic demand of hypertrophied cardiomyocytes. Insufficient microvascular adaptation to cardiomyocyte hypertrophy has been identified as a key pathophysiological feature in heart failure.[12, 13] Indeed, the transition from compensatory hypertrophy to heart failure is associated with down regulation of angiogenic factors and consequent inhibition of microvascular growth.[14] Moreover, inhibition of angiogenesis accelerates the progression of heart failure while stimulation of angiogenesis restores myocardial dysfunction.[14-16] Therapies that restore the cardiac microvasculature might therefore significantly improve cardiac function and potentially morbidity and mortality as well. Although EPO has previously been considered an exclusive hematopoietic hormone, functional EPO-receptors have been detected in several other organs, including the heart.[17] EPO has been shown to salvage myocardial tissue during experimental myocardial infarction.[18] Moreover, EPO consistently restores microvascular insufficiency and improves cardiac function in experimental models of CHF.[19, 20] EPO might therefore have an important therapeutic potential in CHF, beyond the regulation of erythropoiesis. However, the mechanisms of EPO-induced neovascularization in CHF are not well described. It is unknown whether these ancillary cardiac effects are mediated through stimulation of endothelial progenitor cells in the bone marrow, through direct effects on cardiac cells or alternatively represent an epiphenomenon of increasing hematocrit levels.

Aim of the thesis

The aim of the present thesis is twofold:

1 In the first part of the thesis we evaluate the role of **endogenous EPO** in the pathophysiology of anemia in CHF.

In **chapter 2**, we review the biological function of EPO in the control of red blood cell production and introduce the extra-hematopoietic EPO-receptor system. In **chapter 3**, we evaluate whether anemia is related to impaired production of EPO as a result of impaired kidney perfusion. In **chapter 4**, we evaluate whether hematopoietic progenitor cells of CHF patients exhibit impaired sensitivity to EPO and whether this contributes to anemia. In **chapter 5**, we summarize our findings and postulate a model which explains how anemia is related to impaired cardiac performance.

2 In the second part of the thesis we evaluate the mechanisms through which **exogenous EPO** restores cardiac microvascularization anf function in chronic heart failure.

In **chapter 6**, we evaluate the role of bone marrow derived endothelial progenitor cells. In **chapter 7**, we investigate whether the cardiac effects of EPO are in part attributable to the presence of ischemia. In **chapter 8**, we evaluate whether the cardiac effects of EPO are attributable to elevated hematocrit levels. In **chapter 9**, we focus on the effect of EPO on angiogenic factors in the heart.

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20. Westenbrink BD, Voors AA, Ruifrok WP et al. Therapeutic potential of erythropoietin in cardiovascular disease: erythropoiesis and beyond. *Curr Heart Fail Rep* 2007 September;4(3):127-33.

PART 1

Pathophysiology of anemia in heart failure

Chapter 2

Therapeutic potential of erythropoietin in cardiovascular disease: erythropoiesis and beyond

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Willem-Peter T. Ruifrok
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Abstract

Erythropoietin (EPO) is a glycoprotein hormone implicated in the regulation of red blood cell production. Anemia is common in chronic heart failure (CHF) patients and associated with an inappropriately low EPO-production, suggesting a role for its recombinant human form (rhEPO) in treatment. Although safety concerns have been raised regarding treatment with rhEPO in patients with chronic kidney disease, treatment with rhEPO in patients with CHF has so far been safe and well tolerated.The effect of rhEPO on outcome in anemic CHF patients is under investigation in a phase III clinical trial. In addition to its erythropoietic effects, EPO has been detected in the cardiovascular system, fueling intense research into possible non-hematopoietic effects. EPO has been shown to exert protective effects on the heart during acute myocardial ischemia and improve cardiac function in experimental CHF. Acute protection is mediated through reduction of apoptotic cell death. Improvement of cardiac function in CHF is related to myocardial neovascularization. EPO thus exhibits a vast array of beneficial effects in cardiovascular disease. In addition to the correction of anemia in CHF, rhEPO might therefore also benefit patients with cardiovascular disease.

Introduction

Erythropoietin (EPO) is a hematopoietic hormone, and its recombinant human form (rhEPO) has provided a breakthrough in the treatment of anemia caused by EPO deficiency in chronic kidney disease. Over the past decade the indication for rhEPO has markedly broadened and now also includes anemia in patients with cancer who are receiving chemotherapy, patients with HIV who are treated with zidovudine, and treatment of myelodysplastic syndromes. rhEPO treatment is also granted before major surgery as a prophylaxis to reduce blood transfusions or for patients who are unwilling to receive blood. Moreover, treatment of anemia with rhEPO in patients with chronic heart failure (CHF) seems safe and well tolerated, leading to a phase III clinical trial currently enrolling patients. Recently however, a functional EPO-receptor (EPOR) was detected outside the hematopoietic system, fuelling intense research into possible non-hematopoietic effects. From this, EPO has emerged as a myocardial survival and vascular growth factor with a promising protective potential in the setting of acute and chronic myocardial ischemia. This review focuses on the cardiovascular EPO-EPOR system and the potential role of treatment with rhEPO in cardiovascular patients with or without anemia.

Erythropoietic effects of EPO

EPO is a glycoprotein hormone mainly implicated in the regulation of red blood cell production. EPO is produced in the fetal liver and the adult kidney under the transcriptional control of hypoxia inducible factor-1α (HIF-1α).[1] Although EPO is critical for the regulation of erythropoiesis, the production of EPO is not stimulated by diminished red blood cell numbers but instead through the downstream effect on tissue oxygen supply. Under normoxic conditions the continuous production of HIF-1α is antagonized by immediate degradation in the proteasome, making it functionally inactive. Hypoxia directly inhibits proteasomal degradation, resulting in exponentially increased expression of HIF-1α and transcription of a variety of hypoxia responsive genes including EPO. The renal medulla is especially sensitive to alterations in partial oxygen tension, which, together with the ability to rapidly increase EPO levels and exponentially augment red blood cell production, allow for tight regulation hemoglobin (Hb) levels and consequent tissue oxygen supply.

EPO targets a transmembrane member of the cytokine type 1 receptor super-family, ubiquitously expressed on erythroid progenitor cells. Binding of EPO results in dimerization of EPOR, which in turn activates the receptor associated janus tyrosine kinase-2 (JAK2). JAK2 further propagates the signal by engaging secondary signal transduction molecules, including transducers and activators of transcription (STAT), mitogen-activated protein kinases (MAPK), and phosphatidylinosol 3-kinase (PI3K)-protein kinase B pathways. The principal effect of EPO is the reduction of physiologic apoptosis associated with cell turnover in erythroid progenitor cells but

in conjunction with other growth factors EPO additionally stimulates proliferation and differentiation of these cells.[2]

Erythropoiesis in CHF

Anemia (as defined by the World Health Organization as Hb levels < 13 g/dL in men and <12 g/dL in women) is commonly observed in patients with CHF.[3,4] Anemia causes chronic volume overload to the left ventricle which results in increased oxygen consumption, left ventricular (LV) dilatation, and LV hyperthrophy, thereby negatively affecting cardiac function in CHF. Indeed, the presence of anemia in CHF has been consistently associated with impaired survival.

The exact etiology of anemia in CHF has not been fully resolved and is likely to be multifactorial within the population as well as within each patient.[3,4] In contrast to patients with chronic kidney disease, circulating EPO levels are elevated in patients with CHF, increase with the progression of disease, and independently predict impaired survival.[5] However, when EPO levels are corrected for the prevailing Hb by calculating the observed/predicted (O/P) EPO ratio, the vast majority of patients with CHF display signs for insufficient EPO production. Opasich et al.[6] demonstrated that over 90% of anemic CHF patients display significantly depleted O/P EPO ratios compared with healthy controls, which was recently confirmed by our group.[6] The impaired EPO production is caused by a combination of decreased renal function and a direct inhibition of EPO production in the kidney by pro-inflammatory cytokines and angiotensin converting enzyme inhibitors.[4] On the other hand, the relatively elevated EPO levels in CHF are indicative of reduced responsiveness of erythropoietic cells to EPO. Experimental evidence for reduced erythropoiesis in CHF was reported by Iversen et al.[6] In mice with heart failure after myocardial infarction (MI), the erythropoietic progenitor pool was reduced by 40%, apoptosis was increased and proliferation of these cells markedly impaired. This was associated with increased tumor necrosis factor (TNF)-α /Fas expression and increased cytolytic activity of bone marrow natural killer cells, suggesting an important role for inflammation in erythropoiesis inhibition. Interleukin-1, TNF-α and interferon α, β, and γ directly inhibit the formation of mature erythropoietic cells from erythropoietic progenitors in the bone marrow.[9] CHF is frequently associated with elevated levels of pro-inflammatory cytokines, and markers for inflammation are independently related to elevated EPO levels.[7,10] Moreover, we have recently demonstrated that anemia in CHF is partially explained by elevated levels of AcSDKP, a negative regulator of hematopoietic stem cells.[11] The inhibitory effects of inflammatory cytokines and AcSDKP might increase the EPO levels required to maintain adequate red blood cell production. In addition to inhibition by circulating factors, erythropoietic progenitor cells of patients with CHF might exhibit impaired function. Hence, CHF is not primarily associated with defective EPO production but rather an inability to adjust production to an increased demand.

Additional mechanisms may contribute to the development of anemia in CHF. We recently demonstrated that in addition to blunted EPO production and impaired renal perfusion, anemia in CHF is associated with enhanced fluid retention.[7] Fluid retention can result in expansion of plasma volume and consequent hemodilution which may cause pseudo-anemia. Furthermore, CHF is infrequently associated with biochemical indices of impaired iron supply, but iron stores in the bone marrow are significantly depleted.[12] Although this might indicate systemic iron deficiency, it is more likely caused by diversion of iron to the reticuloendothelial system as part of the anemia of chronic disease.[13] Finally, the vast majority of patients in CHF use platelet aggregation inhibitors or anticoagulants, which might cause chronic microscopic blood loss. The extent to which this plays a role in CHF is not well described.

The discovery of a cardiovascular EPO-EPOR system

The first evidence for a biologic role for EPO outside the hematopoietic system came from tissue expression studies in 1992 which demonstrated EPO messenger RNA in the brain.[14] In addition to the adult kidney and the fetal liver, expression of EPO and EPOR has now been detected in the brain, heart, reproductive organs, and endothelial and vascular smooth muscle cells.[15] In contrast to the ubiquitous high expression on erythroid cells, expression of EPOR in non-hematopoietic tissues is relatively low.[15] Expression of both EPO and EPOR, however, rapidly increase following hypoxia and a number of other metabolic stressors including pro-inflammatory cytokines, hypoglycaemia, and increased reactive oxygen species. In addition, EPOR expression is stimulated by rhEPO, most notably in conjunction with hypoxia.[17] Hence, while healthy non-hematopoietic tissues are relatively insensitive to EPO, an ischemic insult will results in autocrine/paracrine production of EPO and increased sensitivity of target cells.

Research into the endogenous significance of this extra-hematopoietic EPO-EPOR system has been hampered by the pivotal importance of its hematopoietic counterpart. EPO and EPOR knockout mice die at 14 weeks gestation due to severe anemia and, in addition, exhibit ventricular hypoplasia and a markedly impaired vascular development.[18] These findings have been interpreted as proof that (cardio) vascular EPOR is crucial for the development of the cardiovascular system, but recent evidence has abrogated this hypothesis.[19] Suzuki et al. developed a murine model in which EPOR expression is restricted to the erythropoietic lineage, by targeted knock-in of the EPOR gene ligated to the GATA-1 promoter, a transcription factor exclusive to erythroid lineage cells. This results in mice that exclusively express the EPOR in erythroid lineage cells while the other organs are devoid of an EPOR. Surprisingly, despite the absence of an EPOR in the cardiovascular system, these mice develop normally and are fertile, indicating that the cardiovascular EPO-EPOR system is dispensable for normal development.[19] Nevertheless, four outstanding publications by

the same group revealed that these mice exhibit markedly increased susceptibility to acute and chronic cardiovascular disease, including more extensive MIs after ischemia reperfusion injury,[20] an impaired angiogenic response to femoral artery occlusion,[19] augmented pressure overload–induced LV dysfunction,[22] and accelerated hypoxia induced pulmonary hypertension.[23] The same group demonstrated a correlation between high serum EPO and smaller myocardial infarct size in patients, interpreted as proof of a possible endogenous protective mechanism.[24] The latter findings should, however, be interpreted with caution, as higher EPO levels were associated with lower Hb levels, and the appropriate multivariable corrections were not made. More compelling evidence was recently published by Ferrario et al. who demonstrated an Hb-independent increase in EPO production after MI, persisting until 7 days after MI.[25] It is unknown whether the increased circulating EPO levels result from cardiac EPO production, although increased EPO-production after MI has been demonstrated in the murine heart. Together these results indicate the existence of an endogenous EPO-EPOR system as part of an endogenous defense mechanism against a broad spectrum of acute and chronic cardiovascular disease.

EPO treatment for acute myocardial ischemia

The anti-apoptotic effects of EPO are crucial for the regulation of red blood cell production but might become equally important for the treatment of acute myocardial ischemia. Following an acute MI, extensive myocardial apoptosis ensues which in part determines the extent of the permanent myocardial damage. EPO exerts potent anti-apoptotic effects in a number of cellular systems including cultured endothelial cells and neonatal rat cardiomyocytes.[15] Moreover, EPO prevents apoptosis from a number of sources, but hypoxia is most extensively studied. The signal transduction pathways associated with the anti-apoptotic actions of EPO in extra-hematopoietic cells display remarkable similarities to erythroid cells including PI3K-AKT, STAT, and MAPK. Research has however in part been restricted to these pathways.[23] It is widely accepted that cytoprotection by EPO is caused by its anti-apoptotic effects. Nevertheless, EPO has been linked to an attenuated inflammatory response of cardiomyocytes, associated with upregulation of endothelial nitric oxide synthase (eNOS).[26] Interestingly, the anti-apoptotic effects of EPO are abrogated by specific eNOS blockers or in cells derived from eNOS knockout mice, suggesting that eNOS upregulation is crucial for the anti-apoptotic effects of EPO as well.[27] The activation of the eNOS pathway might suggest that in addition to the anti-apoptotic effects, systemic EPO treatment might improve endothelial function and consequently reduce peripheral resistance.

Numerous studies have translated the cytoprotective in vitro effects into ex and in vivo models of acute myocardial ischemia reperfusion injury and permanent coronary artery ligation.[23] In the first in vivo study, Calvillo et al. administered a high dose of EPO (5000 U/kg) for 7 consecutive days after myocardial ischemia reperfusion injury in rats, which resulted in a 50% reduced loss of cardiomyocytes

and significantly preserved cardiac function, but to a 30% increased hematocrit as well.[28] Although this study was unable to fully separate cardioprotective effects from the hematopoietic effects, other studies have proven the hematocrit independent nature of cardioprotection.[29] EPO exerts protective effects in models of ischemia/reperfusion injury and permanent coronary artery ligation in mice, rat, rabbits and dogs, with doses ranging from 8000 U/kg to 100 U/kg.[29] Moreover, protection is induced when EPO is administered before ischemia, during ischemia, and at the onset of reperfusion, providing a broad window opportunity.[30] Finally, the effects of a single dose of EPO on infarct size and cardiac function are still present 9 weeks later.[31] Together these experimental data suggest a promising role for rhEPO as a cardioprotective agent in the setting of an acute MI.

EPO treatment in CHF

Irrespective of epicardial coronary anatomy, perfusion of the myocardium is impaired in heart failure due to disproportionate cardiac hyperthrophy relative to (micro) vascular growth, resulting in low-grade ischemia.[32,33] In addition to the negative inotropic effects of impaired perfusion, ischemia will result in a switch from fast energy consuming α-myocin heavy chain (MHC) isoforms to slow β-MHC isoforms, which further impairs contractility.

Therapies aimed at improving cardiac microvascularization might improve cardiac function in CHF. EPO has been shown to stimulate neovascularization by promoting proliferation and survival of endothelial cells in vitro and stimulating angiogenesis in vivo.[34-36] In addition, EPO induces the proliferation, differentiation, and adhesion of a subset of bone marrow derived progenitor cells with an endothelial phenotype (endothelial progenitor cells [EPC]) in vitro and results in marked mobilization of EPC in vivo.[37-39] EPC specifically home to sites of neovascularization and contribute to the formation of new vessels.[40] In order to evaluate the effects of EPO on cardiac function and neovascularization, we induced heart failure in rats by coronary artery ligation and treated them with a high dose (40 mg/kg/3 weeks) of the long acting EPO analogue darbepoetin alfa, starting 3 weeks after MI.[31] Although this delayed treatment did not result in a reduction of infarct size measured after 9 weeks of treatment, cardiac function was significantly improved. The improved cardiac function was associated with increased capillary density and increased capillary:myocyte ratio, indicating neovascularization.

The beneficial effects of EPO on cardiac function and microvascularization in post-MI LV dysfunction have recently been confirmed by three independent studies.[41-43] Furthermore, in a distinct model of chronic myocardial dysfunction, EPO prevented doxorubicin induced deterioration of cardiac function which was also associated with neovascularization of the myocardium.[44,45] The EPO-induced neovascularization is consistently associated with increased circulating EPC. Interestingly, in a model of doxorubicin induced myocardial dysfunction, infusion

of isolated EPC resulted in improved cardiac function and neovascularization in a magnitude equal to EPO.[44] We recently investigated the contribution of EPC to EPO induced neovascularization by replacing bone marrow of rats with genetically labeled cells, which allows differentiation between EPC mediated neovascularization and in situ proliferation of endothelial cells.[46] This study revealed that approximately 30% of the new vessels were comprised of bone marrow--derived cells indicating that EPC contribute to EPO-induced neovascularization. The remaining vessels however comprised of in situ proliferated myocardial endothelial cells, associated with a fivefold increased expression of vascular endothelial growth factor (VEGF). EPO-induced neovascularization in post-MI heart failure therefore seems mediated through a combination of EPC recruitment from the bone marrow and increased myocardial expression of VEGF.

The dosing regimens used in previous studies all resulted in a significant increase in hematocrit levels. When applied to the clinical situation, this could lead to hypertension, seizures, vascular thrombosis, and death, possibly related to abruptly increased hematocrit levels.[47] Therefore, we recently compared the effects of a high EPO dose with a low-dose that had no effect on hematocrit. Similar to high-dose EPO, low-dose treatment resulted in slightly less pronounced but statistically significantly improved cardiac function and improved myocardial microvascularization (unpublished data). Another option to avoid the potentially negative effects of chronic EPO therapy on hematocrit values could be the use of recently discovered non-erythropoietic derivates of EPO, retaining the tissue protective property, without the undesired effect on erythropoiesis.[48] The possibility to separate the erythropoietic and tissue-protective effects is explained through structural differences between receptors in bone marrow and in "peripheral" tissues.[49] Two independent studies have demonstrated that these non-erythropoietic EPOs retain their cardioprotective potential in models of acute MI.[50,51] It is uncertain whether these new EPOs will improve cadiac function in CHF.

Clinical perspectives

The observation that anemia in CHF is associated with defective EPO production has prompted the evaluation of rhEPO for its treatment. Several safety and efficacy studies have evaluated correction of anemia in CHF with rhEPO with or without concomitant administration of intravenous iron. The first study was performed by Silverberg et al. who randomized 32 patients with mild anemia to receive either rhEpo and intravenous iron or no additional treatment, resulting in significant improvement in New York Heart Association functional class and cardiac function.[51] These findings have been corroborated by several randomized, double-blind, placebo-controlled studies. In addition to the amelioration of anemia, EPO treatment improved LV ejection fraction, renal function, exercise capacity and quality-of-life scores and reduced natriuretic peptides and hospital admissions for CHF.[3] A pooled analysis of the two largest studies,

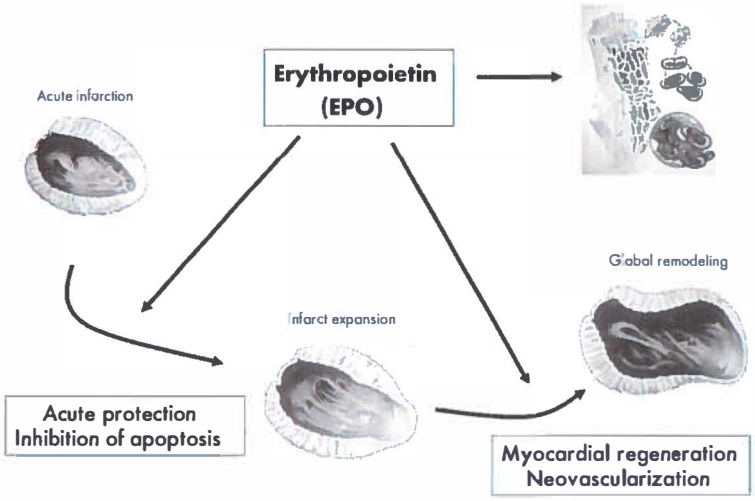
which combined the data of more than 320 patients, revealed a trend to reduced risk of the combined endpoint of CHF-related hospitalization and all-cause mortality in the rhEPO-treated groups.[53] Based on these findings, a large multicenter, double-blind, randomized, placebo-controlled trial was designed, which has recently started enrolling patients (RED-HF). On the other hand, the recently published CHOIR study in patients with chronic kidney disease, demonstrated that normalization of Hb values to reference ranges (Hb > 13.5 g/dL) with rhEPO resulted in significantly more cardiovascular events compared to target levels to 11.3 g/dL, confirming previous findings from Besarab et al.[47,54] These results have intensified the debate on the safety of rhEPO. As a result, the Food and Drug Adminstration has recently recommended "the lowest possible dose to slowly raise the hemoglobin concentration to the lowest level that will avoid the need for a blood transfusion." Of note, the possible beneficial effects of rhEPO in chronic kidney disease patients seem to be distinctly different from patients with CHF. So far, no deleterious effects of rhEPO have been observed in patients with CHF, but current trials will be carefully monitored.

Besides clinical CHF trials, two phase II trials are running in patients with an acute MI. In this setting, only one bolus of EPO is used, and therefore the risks of an unwanted hematocrit-elevation are very limited. We recently performed a randomized safety and feasibility study with a single 300 μg bolus of the long acting EPO analogue darbepoetin alfa, administered during primary percutaneous coronary intervention for a first acute MI. EPO treatment was both safe and well tolerated, caused only a small but nonsignificant increase in hematocrit levels and significantly increased circulating EPCs.[55] These findings led to the design of a randomized multicenter study that evaluates whether EPO can attenuate post-MI loss of cardiac function, currently enrolling patients at our center (NCT00449488). The similar REVEAL study is currently performed in the US (NCT00378352). The results of these studies are clearly awaited.

Conclusions

Although recently scrutinized for other indications, no deleterious effects of rhEPO have been observed in anemic patients with CHF. The efficacy of rhEPO for the correction anemia in CHF is currently under investigation. Recent evidence has however transformed EPO from a designated erythropoietic growth factor to a cytokine that is now recognised for its pleiotropic tissue protective properties. The endogenous EPO-EPOR system is crucial for protection against acute and chronic myocardial ischemia and systemic administration of rhEPO has promising beneficial effects in acute and chronic cardiac disease. Whereas the setting of an acute MI might benefit most from the EPO-induced protection against apoptotic cell death, the chronically failing heart seems to improve through EPO-induced neovascularization (Fig. 1). Therefore, in addition to the correction of anemia in CHF, rhEPO might benefit patients with cardiovascular disease through additional hematocrit independent mechanisms.

Figure 1. Biologic functions of erythopoietin after myocardial injury-erythropoiesis and beyond.



Clinical trial acronyms

CHOIR-Correction of Hemoglobin and Outcomes In Renal insufficiency; RED-HF-Reduction of Events with Darbepoetin alfa in Heart Failure; REVEAL-Reduction of infarct Expansion and Ventricular remodeling with Erythropoietin After Large myocardial infarction.

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Chapter 3

Anemia in chronic heart failure is not only related to impaired renal perfusion and blunted erythropoietin production, but to fluid retention as well

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Abstract

Aims

Anemia is prevalent in the chronic heart failure (CHF) population, but its cause is often unknown. The present study aims to investigate the relation between anemia, renal perfusion, erythropoietin production, and fluid retention in CHF patients.

Methods and results

We studied 97 patients with CHF, of which 15 had anemia (Hb,13.0 g/dL in men and Hb,12.0 g/dL in women), without hematinic deficiencies. Glomerular filtration rate (GFR) and extracellular volume (ECV) were measured as the clearance and the distribution volume of constantly infused ^{125}I-iothalamate, respectively. Effective renal plasma flow (ERPF) was determined as the clearance of ^{131}I-hippuran. Anemic CHF patients displayed significantly reduced GFR (P=0.002), ERPF (P=0.005) and EPO production (P = 0.001), and an elevated ECV (P=0.015). Multivariable analysis demonstrated that lower GFR (P=0.003), lower ERPF (P=0.004), lower EPO production (P=0.006), and a higher ECV (P=0.001) were significant independent predictors of lower hemoglobin levels.

Conclusions

Anemia in CHF is not only independently associated with impaired renal perfusion and blunted EPO production, but to fluid retention as well.

Introduction

Anemia is present in a substantial part of the chronic heart failure (CHF) population, ranging from 15–55%, depending on the definition of anemia and severity of disease.[1]

Anemia is independently associated with increased morbidity and impaired prognosis, although the cause of anemia is frequently unknown.[1–5] CHF is associated with elevated levels of erythropoietin (EPO), suggesting impaired erythropoietic activity in the bone marrow.[1,6,7] Recently, we demonstrated that anemia in CHF could partly be explained by increased serum levels of AcSDKP, a negative regulator of hematopoietic stem cell proliferation.[8] In addition, we hypothesize that CHF will compromise renal perfusion resulting in impaired EPO production, thereby causing anemia. Finally, it has been suggested that anemia in CHF may be partly explained by fluid retention and consequent haemodilution.[9] However, the relative contribution of renal perfusion, EPO production, and fluid retention to the presence of anemia in CHF has so far not been well described. We therefore evaluated the relation between anemia, effective renal plasma flow (ERPF), EPO production, and extracellular volume (ECV) in CHF patients.

Methods

Patient population

Clinically stable CHF patients on outpatient follow-up at our department were asked to participate, as described in detail previously.[10] Approximately 121 patients were asked to participate. In total, 110 patient were included into the original analysis and finished the study. Owing to missing hemoglobin levels, 13 patients were excluded form analysis, leaving 97 subjects for analysis. Briefly, inclusion criteria were age. 18 years and left ventricular ejection fraction (LVEF), 45%. All patients used renin-angiotensin system blockers, and medication had remained stable for at least 1 month. Exclusion criteria included stroke, myocardial infarction, or cardiac revascularization procedures within the last 3 months or scheduled for these procedures, unstable angina, primary renal disease, prior organ transplant, or chronic use of renal function compromising medication.

Cardio-renal haemodynamic parameters

LVEF was determined by nuclear ventriculography or echocardiography using Simpsons rule. Mean arterial pressure (MAP) was calculated from systolic and diastolic blood pressure measurements obtained immediately before ^{125}I-iotholamate and ^{131}I-hippuran clearance measurements from 10 consecutive measurements in supine position using an automated system. N-terminal proBNP (NT-proBNP) was determined by electrochemiluminescence immunoassay on the Roche Elecsys (Roche diagnostics, Netherlands). Glomerular filtration rate (GFR) and ERPF were measured

by constant infusion of the radiolabelled tracers ^{125}I-iothalamate and ^{131}I-hippuran.[11] Briefly, after drawing a blank blood sample, a priming solution containing 0.4 mL/kg body weight of the infusion solution (0.04 MBq of ^{125}I-iothalamate and 0.03 MBq of ^{131}I-hippuran) plus an extra amount of 0.6 MBq of ^{125}I-iothalamate was given at 8 a.m., followed by infusion at 12 mL/h, adapted to 9 mL/h in subjects with renal function impairment as estimated from previously obtained serum creatinine values. This ensures steady-state plasma levels of ^{131}I-hippuran and ^{125}I-iothalamate after a run-in period of 2 h, as verified by hourly blood samples. Subsequently, clearances of ^{125}I-iothalamate and ^{131}I-hippuran and the distribution volume of ^{125}I-iothalamate were measured during steady state. The GFR and ERPF were calculated as $(U.V)/P_{(iothalamate)}$ and $(I.V)/P_{(hippuran)}$, respectively, and $(U.V)/P_{(iothalamate)}$ was corrected for voiding errors by the ratio of the urinary to plasma clearance of ^{131}I-hippuran. U.V represents the urinary excretion of the tracer and I.V the infusion rate of the tracer; P represents the values in plasma calculated from the samples bracketing each clearance period. The body surface area (BSA) was calculated as $0.007184.weight^{0.425}.length^{0.725}$, and GFR and ERPF were expressed per 1.73 m2 of BSA. Renal blood flow (RBF) was calculated as ERPF/1-haematocrit. The filtration fraction (FF) was calculated as the ratio of GFR and ERPF and expressed as percentage. ECV was estimated from the distribution volume of ^{125}I-iotholamate[12,13] and calculated as $[(I.V + B.V) - U.V]/ P^{iothalamate}$ during steady state. B.V represents the bolus infusion of the tracer. GFR and ERPF were expressed per 1.73 m^2 of BSA, and ECV was expressed as L/kg body weight.

Hemoglobin levels, haematinic parameters, and EPO levels

Hemoglobin, iron, ferritin, transferrin, and vitamin B11 and B12 levels were determined at the local laboratory facilities. EPO levels were determined by IMMULITE EPO assay (DPC, Los Angeles, CA, USA). To define the relation between EPO levels and a given Hb, we included 15 reference subjects referred to our department with complaints of chest pain or palpitations. The reference subjects had a mean age of 50 ± 4.5 years and had normal LVEF (LVEF > 60%), normal renal function, no signs of inflammation, or symptoms of CHF.[14] An exponential regression equation of serum EPO vs. Hb (mmol/L) was calculated, resulting in the following equation log EPO = 3.015 -(0.130 * Hb). Predicted log EPO and observed/ predicted log EPO ratio (log serum EPO/predicted log EPO) were calculated with this equation. Mean O/P ratio in reference subjects was 0.90±0.029 (95% CI 0.64–1.12). Total iron binding capacity (TIBC) was calculated by multiplying serum transferrin x 20. Transferrin saturation (FeSat) was calculated as (serum iron/TIBC) * 100%. Iron deficiency was defined as ferritin levels ,30 mg/L or FeSat, 15%. According to local laboratory reference ranges, deficiency in vitamin B11 and B12 was defined as levels, 142 pmol/L and 50 pg/mL, respectively.

High-sensitivity CRP was determined by nepholometry. The threshold for detection was 0.156 mg/L; when CRP levels below the detection limit, were assigned the value 0.156 mg/L for statistical purposes.

Statistics

Data are given as mean ± standard deviation when normally distributed, as median and interquartile range when skewed distributed, and as frequencies and percentages for categorical variables. Differences between groups were compared with Student's t-test, Mann–Whitney U test, x2 of Fisher's exact test where appropriate. A P-value, 0.05 was considered statistically significant, and all reported probability values are two-sided. Correlation between Hb, EPO or O/P ratio, and various other variables was performed using Pearson's correlation coefficients. Non-normally distributed continuous variables were log-transformed. The variables age, sex, pharmacological treatments, NewYork Heart Association (NYHA) functional class, LVEF, ERPF, GFR, FF, ECV, NT-proBNP, CRP, and MAP were assessed for univariate linear association with Hb or log EPO. Variables that showed a significant ($P < 0.15$) univariate association were included stepwise in a multivariable linear regression model on the basis of on the strength of the univariate association. All the variables described earlier were added to the final model simultaneously to assure that addition of these variables did not significantly increase the predictive accuracy of the model. The final model was assessed for first line interaction.

Results

Patient characteristics

Seventy six percent of subjects were male and age ranged from 27 to 81 years. NYHA functional classes I, II, III, and IV comprised 14, 44, 31, and 10% of patients, respectively.

Difference in characteristics between anemic and non-anemic CHF patients

In the total population, 19 patients (20%) were anemic according to the WHO criteria (Hb ,13.0 g/dL in men and Hb ,12.0 g/dL in women). Iron deficiency was present in 4 out of 19 anemic (21%) and 3 out of 78 non-anemic (4%) CHF patients. Other hematinic deficiencies were not observed. The iron-deficient patients were excluded from further analysis, leaving 75 non-anemic subjects and 15 subjects with unexplained anemia. Differences in characteristics between anemic and non-anemic subjects are summarized in Table 1. Anemic subjects were significantly older and in a higher NYHA class. Although LVEF was comparable, compared with non-anemic patients, anemic patients showed more severe haemodynamic impairment, reflected by reduced MAP (86.8 ± 13 vs. 76.8 ± 14 mmHg; P=0.007), ERPF (286 ± 83 vs. 219 ± 74 mL/min/1.73 m2; P=0.005) and RBF (502 ± 150 vs. 348 ± 121 mL/min/1.73 m2; P<0.001), and elevatedNT-proBNP levels [10 (260–1355) vs. 1004 (720–1904) pg/mL; P=0.029]. Anemic CHF patients were more often using diuretics (65 vs. 87%; P=0.045) and despite this displayed significantly elevated ECV (0.25 ± 0.5 vs. 0.29 ± 0.4 L/kg; P=0.015), implicating fluid overload.The fluid retention was subclinical,

Table 1. Characteristics of patients with unexplained anemia and non-anemic CHF patients.

	Non-anemic (n= 75)	Anemic (n=15)	P-value
Age (years)	56.3 ± 12	65.6 ± 9	0.004*
Sex (n, % male)	58 (85)	10 (71)	0.510
NYHA class	2.3 ± 0.8	2.8 ± 0.8	0.036*
BMI (kg/M2)	27.8 ± 4	26.1 ± 3	0.140
Ischemic etiology (n, %)	38 (50)	7 (47)	1
Cardiorenal hemodynamic parameters			
Heart rate	65 ± 2	64 ± 3	0.834
MAP (mmHg)	86.8 ± 1.5	76.8 ± 3.6	0.007*
LVEF (%)	28 ± 10	26 ± 6	0.525
NT pro-BNP (pg/mL)	510 (260-1355)	1004 (720-1904)	0.029*
Creatinine (mg/dL)	1.2 (1-2)	1.2 (1.1-1.7)	0.150
Urea (mg/dL)	19 (16-22)	35 (23-40)	<0.001*
Plasma sodium (mEq/L)	137 ± 3	136 ± 3	0.589
GFR (mL / min / 1.73 m2)	79 ± 25	56 ± 28	0.002*
ERPF (mL / min / 1.73 m2)	286 ± 83	219 ± 74	0.005*
RBF (mL / min / 1.73 m2)	502 ± 150	348 ± 121	<0.001*
FF (%)	28 (26-30)	26 (20-29)	0.370
FENa (%)	0.88 ± 0.33	0.97± 0.52	0.370
ECV/body weight (L/kg)	0.25 ± 0.5	0.29 ± 0.4	0.015*
Mild RF (GFR <60)	16 (21%)	9 (60%)	0.004*
Severe RF (GFR<30)	4 (5%)	3 (20%)	0.088
Erythropoietic and inflammatory parameters			
Hb (mg/dL)	15 ± 0.7	12.7 ± 0.4	<0.001*
Serum EPO (U/L)	15.7 (11-21)	18.5 (12-31)	0.357
O / P ratio	1.15 ± 0.2	0.93 ± 0.18	0.001*
CRP (mg/L)	2.15 (0.95-4.06)	2.38 (0.77-5.66)	0.733
Medication			
ACE inhibitors, n (%)	66 (88)	12 (80)	1
ARB, n (%)	9 (12)	3 (20)	0.414
Beta blokkers, n (%)	62 (83)	13 (87)	1
Diuretic, n (%)	49 (65)	13 (87)	0.045*
Aldosterone ant. (%)	51 (BB)	7 (47)	0.144

*p<0.05 All continuous variable are presented as mean ± SD, if normally distributed and as median value with 25th - 75th percentile when skewed distributed. NYHA; New York Heart Association functional class, BMI; Body Mass Index, MAP; mean arterial pressure, LVEF; left ventricular ejection fraction, NT pro_BNP; N terminal pro_brain natriuretic peptide, GFR; glomerular filtration rate, ERPF; effective renal plasma flow, RBF; renal blood flow, FF; filtration fraction, ECV; extracellular volume, FENa; fractional Na excretion, Hb; hemoglobin, RF; renal failure, EPO, erythropoietin levels, O / P ration, ratio between observed and predicted LogEPO, CRP; C-reactive protein, ARB; angiotensin receptor blokker, aldosteron ant; aldosteron antagonists.

as anemic patients did not display oedema, nocturia, or dyspnoea more frequently (data not shown). Plasma sodium levels and fractional sodium excretion were similar, implicating that the elevated ECV was not caused by excess sodium intake. Although creatinine levels were comparable between groups, urea levels were elevated [19 (16–22) vs. 35 (23-40) mg/dL; P=0.007] and GFR (79 ± 25 vs. 56+28 mL/min/1.73 m2; P=0.002) was significantly reduced in anemic patients. Moreover, anemic patients had a higher incidence of moderate renal failure (GFR<60 mL/min/1.73 m2) and a trend towards more severe renal failure (GFR, 30 mL/min/1.73 m2) (21 vs. 60%, P<0.005 and 5 vs. 20%, P=0.088, respectively).

By definition, Hb level was significantly lower in anemic subjects. However, EPO levels were comparable between anemic and non-anemic CHF patients. Additionally observed/predicted (O/P) ratio was significantly reduced in anemic subjects (1.15 ± 0.2 vs. 0.93 ± 0.2, P=0.001), indicating blunted EPO production. O/P ratio was significantly higher in non-anemic CHF patients compared with reference subjects (P=0.026), whereas O/P ratio in anemic CHF patients and controls were comparable. Thus, EPO production is elevated in both anemic and non-anemic CHF patients. But based on their Hb, it should have been higher in anemic CHF patients. It therefore seems that the compensatory rise in response to anemia is impaired.

Hb levels and EPO production in the CHF population

CHF patients displayed a relatively moderate negative correlation between EPO and Hb levels (R=-0.281, P=0.007). A moderate significant correlation was also observed between EPO levels and both CRP (R=0.281, P=0.007) and NYHA class (R=0.210, P=0.05), and a trend with NT-proBNP (R=0.193, P=0.07). No significant correlation was observed between EPO levels and other markers for cardiorenal hemodynamic status, or renal function parameters. O/P ratio correlated with Hb (R=0.397, P=0.001), GFR (R=0.237, P=0.024), and FF (R=0.285, P=0.007).

Predictors of Hb and serum EPO

Univariate and multivariable linear associations between Hb and EPO levels are displayed in Tables 2 and 3, respectively. Sex, lower EPO (Figure 1A), lower GFR (Figure 1B), lower ERPF (Figure 1C), and higher ECV (Figure 1D) were independently associated with lower Hb levels, accounting for 31-44% of the variance in Hb levels. The variables CRP, NYHA class, NT-proBNP, and Hb showed significant univariate association with plasma EPO. However, higher CRP and lower Hb levels were the only independent predictors of higher serum EPO levels. Inclusion of the full list of possible predictive variables did not result in a significant increase in the adjusted R^2, slope, or partial correlation coefficient of the variables in our model.

Table 2. Univariate, and multivariable predictors of Hb levels.

Hemoglobin							
Univariate			**Multivariable**				
Variable	B	SE	B	SE	β	Part. cor.	P
Sex	-1.41	0.30*	-1.189	0.258	-0.394	-0.449	<0.001
Age	-0.022	0.012*					
GFR	0.02	0.005*	0.037	0.009	0.744	0.414	<0.001
ERPF	-0.004	0.002*	-0.008	0.003	-0.534	-0.310	0.004
NYHA	-0.04	0.17*					
EPO	-1.55	0.57*	-1.422	0.430	-0.266	-0.399	0.001
ECV	-6.36	2.95*	-7.120	2.215	-0.259	-0.330	0.004
MAP	-0.03	0.01*					
BNP	-0.399	0.144*					
						Adjusted R-square 0.436	

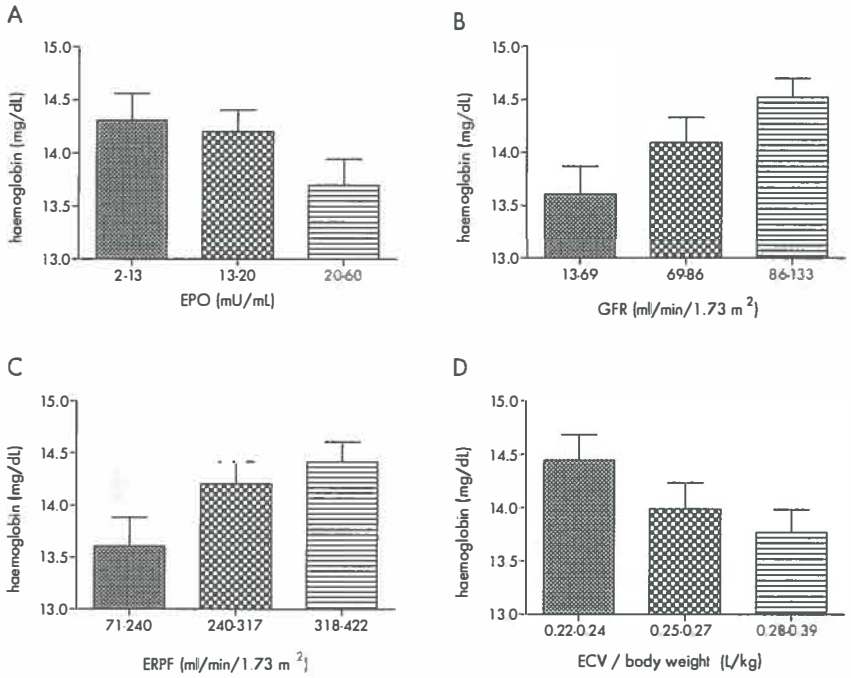
* p<0.15, GFR; glomerulor filtration rate, ERPF; effective renal plasma flaw, NYHA; New York Heart Association functional class, EPO; serum erythropoietin levels, ECV; extra cellular volume corrected for bodyweight, MAP; mean arterial pressure, BNP; N terminal pro_brain natriuretic peptide, β; standardised beta, part. cor.; partial correlation coefficient

Table 3. Univariate, and multivariable predictors of serum EPO levels.

Erythropoietin						
Univariate			**Multivariable**			
Variable	B	SE	B	SE	β	P
Sex	0.03	0.233				
Age	0.027	0.6				
Hb	-0.052	0.019*	-0.044	0.019	-0.233	0.025
NYHA	-0.06	0.031*				
CRP	0.345	0.126*	0.125	0.054	0.234	0.024
				Adjusted R-square: 0.111		

*p<0.15. Hb; hemoglobin levels, NYHA; New York Heart Association functional class, CRP; C-reactive protein

Figure 1. Relation between hemoglobin levels and erythropoietin, GFR, ERPF and extracellular volume.



EPO; erythropoietin, GFR; glomerular filtration rate, ERPF; estimated renal plasma flow, ECV; extracellular volume.

Discussion

The present study demonstrated that anemia in CHF patients was not only independently related to impaired renal perfusion and blunted EPO production, but to an increased ECV as well. However, in contrast to our expectations, serum EPO levels were not directly related to renal perfusion.

The association between anemia in CHF and impaired EPO production has been suggested previously.[3,7] Nevertheless, the presence of defective endogenous EPO production was not formally evaluated until recently. In a comprehensive retrospective analysis on the cause of anemia in CHF patients, Opasich et al.[15] found that 50% of anemic CHF patients showed evidence of impaired EPO production. Our data further substantiate these findings.

The relation between renal perfusion and EPO levels has been evaluated previously in two populations comprising 13 and 14 CHF patients.[16,17] In these studies, EPO production inversely correlated with RBF, ERPF and renal oxygen delivery, suggesting that impaired renal oxygenation caused the elevated EPO levels. However, in our far larger cohort, these findings could not be reproduced. Although there was no relation between ERPF and EPO production, a univariate mild correlation between

EPO production and GFR was observed, which might implicate that blunted EPO production results from impaired renal function and structural renal damage. Furthermore, circulating inflammatory cytokines and ACE-inhibitors can directly inhibit EPO production in the kidney and might contribute to the blunted EPO production.[18,19] Additionally, impaired GFR could attenuate the excretion of circulating erythropoiesis-inhibiting factors (e.g. AcSDKP), leading to enhanced plasma levels, as has been demonstrated in a hemodialysis population.[20]

The non-anemic CHF patients displayed higher EPO levels and O/P ratios than reference subjects, as has been described previously. Elevated EPO levels were independently related to higher CRP levels, suggesting that elevated EPO production is directly related to an enhanced inflammatory state. Several pro-inflammatory cytokines have inhibitory effects on erythropoiesis and are established as the cause of anemia associated with chronic inflammatory disease.[21] CHF is associated with enhanced expression of a variety of pro-inflammatory cytokines, possibly contributing to the development of anemia.[6] In addition, we have recently demonstrated that anemia in CHF could be partially explained by elevated levels of AcSDKP, a negative regulator of hematopoietic stem cells.[8] These circulating factors inhibit erythropoiesis and can eventually result in elevated EPO requirements. Indeed, although EPO production was blunted, the circulating EPO levels in anemic CHF patients were not reduced but slightly elevated compared with non-anemic patients. The slightly elevated EPO levels were however insufficient for the prevailing Hb, reflected by significantly impaired O/P ratio. Hence, anemia in CHF does not result from the inability to produce EPO, but an inability to further increase baseline EPO production.

As expected, anemic patients displayed elevated ECV, which was independently related to lower Hb levels. Impaired renal hemodynamics in CHF cause activation of RAS and vasopressin systems, resulting in salt and fluid retention and consequently increased ECV. Fluid retention in CHF can cause haemodilution, resulting in pseudo-anemia, which carries an even worse prognosis than true anemia.[9] In the present study, anemic subjects more frequently received diuretics but nonetheless displayed elevated ECV. Importantly, although fluid retention was related to anemia, signs and symptoms of fluid retention were absent. Thus, hemodilution seems to precede the clinical presentation of fluid retention. Therefore, starting or increasing the dose of diuretics should be considered before starting with EPO treatment. Since the etiology of anemia in CHF seems multifactorial, the preponderant cause should be identified on an individual basis, for instance, by determining the O/P ratio or ECV in addition to regular diagnostic procedures.

Although the reduced renal perfusion, blunted EPO production, and elevated ECV could be the cause of anemia in CHF, they could also be a consequence. Lower Hb levels can result in peripheral tissue hypoxia, causing vasodilatation and consequently reducing blood pressure.[22] This will result in activation of the RAS and further compromise of RBF by renal vasoconstriction and fluid retention. The compromised kidney seems unable to meet the increased demand, and anemia ensues. The vicious cycle of CHF causing anemia and anemia causing further deterioration of CHF has been described as the cardiorenal anemia syndrome.[23]

Thus, anemia in CHF is directly related to an impaired haemodynamic state, compromising renal perfusion, attenuating EPO production, and increasing fluid retention. Therefore, improvement of cardiac function and cardiorenal hemodynamics would be the most rational approach for the treatment of anemia in CHF. Additionally, administration of recombinant human EPO might break the vicious cycle by replenishing the insufficient EPO levels.[24] It is however uncertain whether supplementation of EPO in anemic CHF patients will decrease morbidity and mortality, as anemia might merely be a marker for impaired cardiac function. This will emerge from scheduled randomized clinical trails. The elevated ECV in our population suggests that concomitant meticulous correction of fluid overload might be feasible. Whether this will improve Hb and outcome in this population is however uncertain.

Limitations

Our study has limitations. Apart from the obvious crosssectional design, the CHF population contained relatively few anemic CHF patients and anemic subjects had relatively mild anemia. Therefore, our data might not be representative for more severe forms of anemia and should be regarded as hypothesis generating.

Conclusions

Anemia in CHF is not only independently associated with impaired renal and blunted EPO production, but to fluid retention as well.

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Chapter 4

Bone marrow dysfunction in chronic heart failure contributes to impaired erythropoiesis

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Abstract

Background

Anemia of unknown etiology is frequently found in chronic heart failure (CHF) patients and predicts a poor prognosis. We hypothesized that bone marrow dysfunction might be the underlying cause, and therefore evaluated erythropoiesis in CHF patients.

Methods

Bone marrow mononuclear cells (MNC) were harvested from the sternum during elective cardiac surgery from 12 patients with CHF (age 68 ± 2; 53% male, Left Ventricular Ejection Fraction (LVEF) 32 ± 1.8 %) and 12 age matched patients with normal left ventricular function (LVEF ≥ 55%). We quantified early hematopoietic progenitor cells ($CD34^+$) and committed erythroid lineage cells ($CD71^{bright}$), apoptosis ($Annexin\text{-}V^+$ / $DAPI^+$) and erythropoietin-receptor (EPO-R) density by flow cytometry. The potential of isolated $CD34^+$ HPCs to form erythroid colonies was assessed in methylcellulose medium containing incrementing doses of EPO. After 14 days, the number of Burst Forming Units Erythroid (BFU-E) were counted.

Results

Fifty percent of CHF patients had anemia of undetermined origin. The erythroid lineage was significantly depleted in CHF patients, reflected by 2-fold lower number of $CD34^+$ and a 30% reduction in CD71 bright cells ($P<0.05$ for both). Throughout the EPO dose-response range, $CD34^+$ cells of CHF patients produced a 3-fold lower number of BFU-E colonies compared to controls ($P<0.02$). Bone marrow apoptosis was 3-fold increased in CHF patients compared to controls ($P<0.01$), while EPO-R density was comparable. Moreover, BFU-E formation did not differ between anemic and non-anemic CHF patients ($P=0.8$). BFU-E numbers were inversely correlated with plasma NTproBNP levels ($R= -0.5$, $P =0.03$), even after adjusting for potential confounders.

Conclusions

CHF causes profound dysfunction of the hematopoietic compartment, reflected by impaired erythropoiesis. Although bone marrow dysfunction might render patients more susceptible to anemia, it does not exclusively explain its occurrence.

Introduction

Chronic heart failure (CHF) is the final common endpoint of the majority of cardiac conditions and represents the leading cause of death in the Western world. Anemia of undetermined origin is frequently found in patients with CHF, and predicts an even poorer prognosis.[1-2] Resolving the preponderant etiology of anemia in these patients is therefore warranted.[3]

An intriguing finding in CHF patients is that erythropoietin (EPO) levels are relatively elevated and the strong and linear association between hemoglobin (Hb) and EPO levels is distorted.[4-6] These findings suggest that hematopoietic cells of CHF patients are relatively insensitive to EPO and may require more EPO to maintain adequate erythropoiesis. The relative resistance to EPO might in part be explained by the anti-proliferative effects of inflammatory cytokines or ACE-modulating drugs in the circulation.[7-10] In addition, CHF patients might exhibit specific cellular EPO-resistance, for instance by down regulation of the EPO-receptor (EPO-R) or its downstream signal transduction pathways in erythroid cells.[11] Interestingly, bone marrow cells of CHF patients have been shown to display significantly reduced myeloid and endothelial progenitor cell colony formation, suggesting that bone marrow function might be generally affected.[12]

We hypothesised that CHF is associated with general bone marrow dysfunction which might in part explain the high incidence of anemia in these patients. An important determinant of adequate hematopoiesis is the clonogenic expansion of hematopoietic progenitor cells (HPCs) into mature hematopoietic cells. We therefore compared the *in vitro* clonogenic potential of isolated $CD34^+$ HPCs from CHF patients with age matched controls and evaluated their relation to the severity of CHF and the presence of anemia.

Methods

Patients

We enrolled symptomatic heart failure patients scheduled for elective cardiac surgery with left ventricular ejection fraction (LVEF) < 0.40 and age > 18 years and their age-matched controls with normal cardiac function (LVEF > 0.55). Exclusion criteria included previous chemotherapy, previous or primary hematological disease, solid tumors within the last 7 years, chronic inflammatory disease, substance abuse, current severe renal failure or organ transplant recipients. The protocol was approved by the local ethical committee and all patients provided written informed consent.

Biochemical analysis of peripheral blood

Hemoglobin (Hb), hematocrit, leucocyte numbers, renal function, N-terminal pro-brain natriuretic peptide (NTproBNP), lactate dehydrogenase (LDH), ferritin, transferin, Iron, vitamin B1 and B12 levels were assessed pre-operatively in the

local laboratory facilities. Erythropoietin (EPO) levels were determined as described previously.[5] Transferrin saturation was calculated as previously described.[6] Iron deficiency was defined as ferritin levels < 20 µg/L or FeSat <10%. According to the local laboratory reference ranges, deficiency in vitamin B11 or B12 was defined as levels < 142 pmol/L and 50 pg/mL, respectively.

Bone marrow samples

Whole bone marrow was recovered from the sternum during surgery and transferred into sterile tubes containing RPMI 1640 medium (Cambrex, NJ, USA) with preservative free heparin. MNCs were isolated from whole bone marrow through density centrifugation with Lymphoprep (Fresenius, Norway) according to suppliers' guidelines.

Flow cytometry

Bone marrow MNCs were incubated with PerCP-labelled anti-human CD34, or APC-labelled anti-human CD71 (BD BioSciences, Erembodegem, Belgium) to evaluate consecutive stages in erythroid differentiation. EPO-receptor (EPOR) density was determined with a Phycoerythrine (PE) labelled anti human EPOR antibody (R&D Systems, USA). EPO-R density was estimated from the mean fluorescent intensity. Variation coefficient throughout the study period for PE measurements was less than 5 % as determined with the Califlow kit (Sferotec, Germany). Early apoptosis in MNCs was evaluated with the with the Annexin-V kit (BENDER med systems, Austria), which entails a FITC labelled anti human Annexin-V (Ann-V) antibody. Diamidino-2-phelylindole (DAPI, 0.5 µg/ml) was used to evaluate the integrity of the cell membrane and identify later stages of apoptosis. Cells were considered early apoptotic if Ann-5$^+$ / DAPI$^-$ and late apoptotic if Ann-5$^+$ / DAPI$^+$. Samples were measured on the LSR-II flow cytometer (Becton and Dickinson, USA) and analysed using Winlist software (version 6.0, Versity Software, USA).

Isolation of hematopoietic progenitor cells

Hematopoietic progenitor cells were isolated on the basis of CD34 expression from bone marrow MNCs using the MiniMACS immunomagnetic magnetic separation system (Militenyi Biotec, Germany) according to the manufacturers guidelines.

In vitro erythopoiesis

Isolated CD34$^+$ cells (10^4) were aliquoted in quadruplicate in Methocult H4230 medium (Stem Cell Technologies, United Kingdom) containing 0.02, 0.1. 0.2, 1, 2 or 10 iU/mL of recombinant human erythropoietin (EPREX, Janssen-Cilag, the Netherlands) and cultured at 37°C and 5% CO_2 in a humidified atmosphere. After 14 days, the number of burst forming units erythroid (BFU-E) colonies were counted. To control for procedural variation, a reference sample comprising of pooled CD34$^+$ cells from 5 healthy controls was cultured in parallel with a single concentration of 2 IU/mL of EPO in each experiment. BFU-E colony numbers were normalized to the reference values and expressed as number of colonies per 10^4 CD34$^+$ cells. In a subset

of cultures from 4 representative subjects per group, cells were resuspended from methylcellulose and counted to obtain the average number of cells per colony.

Statistical analysis

Data are given as mean ± standard errors of the mean (SEM) for continuous variables or as percentages for categorical variables. Differences between groups were compared with students T-test, Mann Whitney-U test, Chi-square test or Fishers exact test, where appropriate. Differences in BFU-E dose-response curves between groups were assessed by ANCOVA for repeated measurements after log-transformation. Correlations between BFU-E formation, bone marrow apoptosis and various other variables were performed using Pearsons correlation coefficients. Non-normally distributed variables were log-transformed. To control for potential confounders, standard linear regression analysis was used to control for age, sex, ACE-inhibitor therapy and Hb levels. P-values below 0.05 were considered to denote a statistically significant difference. Calculations were performed using SPSS-software (version 15).

Results

CHF patients displayed slightly but non-significantly lower hemoglobin levels compared to controls (13 ± 0.5 versus (vs) 14 ± 0.3, p=0.2) and six out of 12 CHF patients were anemic according to the World Health Organisation criteria (Hb < 13 g/dL in men and Hb < 12 g/dl in women). One non-anemic subject had mild iron deficiency, no other hematinic deficiencies were observed in the study population. CHF patients had a higher incidence of previous myocardial infarction (67% vs 9%, P<0.01), were more frequently on loop diuretics (67% vs 17%, p=0.04) and had higher NTproBNP levels (2914 ± 1070 vs 424 ± 256, p<0.001) compared to controls. The indication for surgery, the use of ACE-inhibitors and estimated GFR were similar among groups. Other general characteristics of the study population are presented in table 1.

Erythroid lineage and apoptosis

Both the percentage of $CD34^+$ hematopoietic progenitor cells and $CD71^{bright}$ committed erythroid cells were significantly decreased in CHF patients compared to controls (0.77 ± 0.11 % vs 1.3 ± 0.25 % $CD34^+$ cells, and 21 ± 3.5 % vs 31 ± 2.7 % $CD71^{bright}$ cells P<0.05, respectively, table 2). Moreover, both early and late apoptosis was significantly increased in bone marrow MNCs of CHF patients (6.3 ± 1.6 % versus 2.2 ± 0.7 %, P<0.05 and 3.8 ± 0.7 % vs 1.3 ± 0.2 %, P<0.02 respectively, table 2). Numbers of erythroid cells and apoptotic cells were comparable between anemic and non-anemic CHF patients, although a trend towards further reduction in $CD71^{bright}$ cells was observed in the anemic CHF group (27 ± 3.9 % vs 15 ± 4.3 % $CD71^{bright}$ cells in non-anemic vs anemic CHF patients, P=0.08, table 2).

Table 1. Demographics of the study population.

	Patients and Controls			Anemic and Non-Anemic patients		
Variable	**Controls (n=12)**	**Total CHF (n=12)**	**P**	**Non-anemic CHF (n=6)**	**Anemic CHF (n=6)**	**P**
Hemoglobin (mg / dL)	13.5 ± 0.3	12.8 ±0.5	0.2	13.3 ± 0.5	12.2 ± 0.7	-
Age	68 ± 2	68 ± 2	-	68 ± 4	69 ± 2	0.8
Male gender (n, %)	9 (56)	7 (44)	0.7	3 (43)	4 (57)	0.5
LVEF	58 ± 1.6	32 ± 1.8	<0.001 *	32 ± 2.1	31 ± 3.2	0.9
NTproBNP (pg/mL)	424 ± 257	2913 ± 1070	<0.001 *	2491 ± 1667	3336 ±1481	0.2
Ischemic CHF (n, %)	-	8 (89%)		3 (38)	5 (63)	1
eGFR, mL/min	86 ± 5	80 ± 7	0.5	82 ± 9	79 ± 10	0.8
CABG (n, %)	11 (92)	9 (75)	0.3	3 (50)	6 (100)	0.09
Previous MI (n, %)	1 (11)	8 (89)	0.1	4 (67)	4 (67)	0.7
Diabetes (n, %)	8 (53)	7 (46)	0.4	4 (67)	3 (50	0.5
EPO levels (iU/ml)	13 ± 2	12 ± 2	0.4	14 ± 2	10 ± 3	0.3
Tsat (%)	31 ± 4	25 ± 3	0.2	29 ± 2.8	21 ± 4	0.2
Ferritin, µg/L	179 ± 52	220 ± 32	0.5	243 ± 36	200 ± 55	0.5
Vitamin B12, pmol/L	306 ± 43	373 ± 55	0.3	454 ± 93	292 ± 48	0.2
Folate, nmol/L	30 ± 4	17 ± 2	0.03	19 ± 2	15 ± 3	0.2
Medication, % use						
ACE inhibitors	9 (75)	11 (92)	0.7	6 (100)	5 (80)	0.5
B-blocker	11 (92)	11(92)	0.5	5 (80)	6 (80)	0.5
Aldosterone antagonists	0	4 (33)	0.04*	1 (20)	3 (50)	0.3
Loop diuretics	2 (17)	8 (89)	0.03*	3 (50)	5 (80)	0.3

All variables are presented as mean ± standard error. CHF, Chronic Heart Faiure; NTproBNP, N-terminal pro Brian Natriuretic Peptide; LVEF, left ventricular ejection fraction; eGFR, estimated glomerular filtration rate; MI, myocardial infarction; EPO, erythropoietin; Tsat, % transferrin saturation; ACE-I, angiotensin converting enzyme inhibitor or angiotensin receptor blocker.

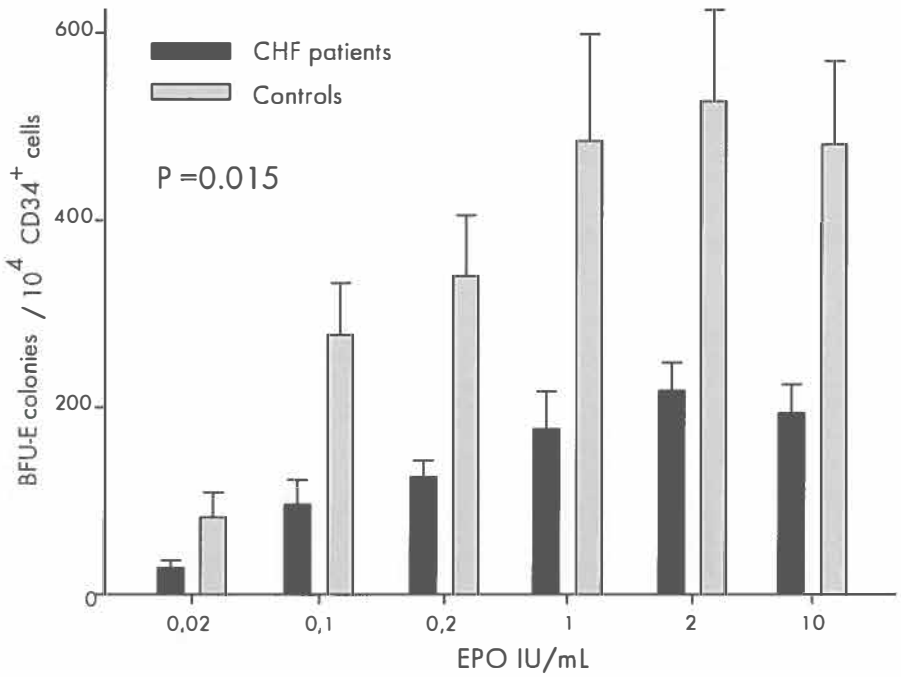
Erythropoietin receptor density

Average EPOR density in EPOR expressing cells was slightly but not significantly higher in CHF patients compared to controls (relative fluorescence intensity 40 ± 3 vs 33 ± 1, P=0.07, table 2). The EPOR density was comparable between anemic and non-anemic CHF patients (40 ± 6 vs 41 ± 3, P=0.1, table 2).

BFU-E formation

Throughout the EPO dose response range, the average number of BFU-E colonies after 14 days of culture was 2-3-fold lower in CHF patients compared to controls (217 ± 30 vs 527 ± 98 colonies per 10^4 $CD34^+$ cells with 2 U/mL EPO, P=0.015 general linear model, Figure 1). The EPO-response of $CD34^+$ cells, evidenced by the relative increase in BFU-E formation to incrementing doses of EPO did not differ between CHF patients and controls (P=0.9, figure 2A).

Figure 1. ***In vitro*** **BFU-E colony formation in CHF patients and age matched controls.**



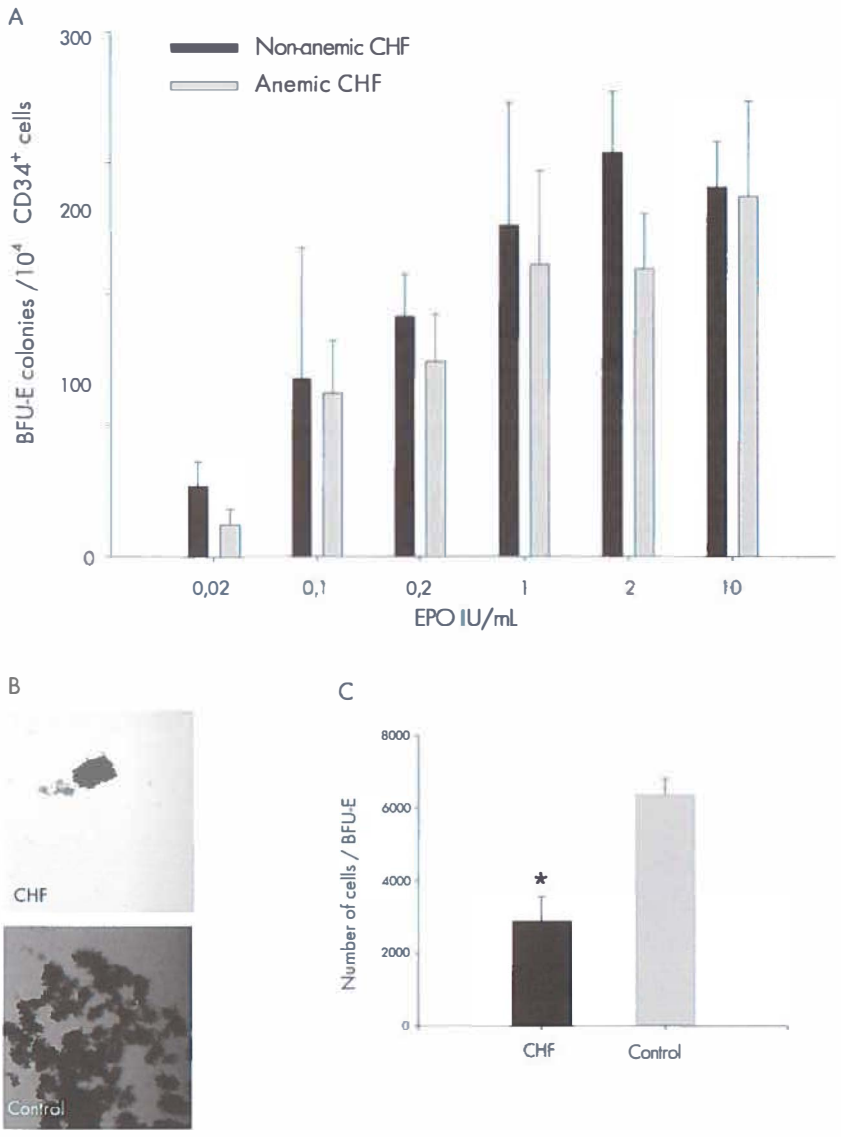
BFU-E, burst forming unit-erythroid; CHF, chronic heart failure.

Table 2. Flow cytometry results.

	Patients and Controls			Anemic and Non-Anemic patients		
Variable	**Controls (n=12)**	**Total CHF (n=12)**	**P**	**Non-anemic CHF (n=6)**	**Anemic CHF (n=6)**	**P**
Hematopoietic progenitor cells(% $CD34^+$)	1.3 (0.8-1.5)	0.7 (0.6-0.9)	0.038*	0.7 (0.7-1.3)	0.6 (0.5 0.9)	0.3
Commited erythroid cells (% $CD71^{-bright}$)	30 (25-40)	21 (13-29)	0.047*	26 (20-35)	14 (7-24)	0.08
Early apoptotic cells (% Ann-5 $^+$ / DAPI $^-$)	1.5 (0.8-3.4)	5.3 (2.9-8.1)	0.01 *	5.3 (4.4-7.8)	4.7 (2-14)	0.9
Late apoptotic cells (% Ann-5 $^+$ / DAPI $^+$)	(0.8-1.7)	3.7 (1.9-5.9)	0.003*	4.5 (2.3-5.9)	2.7 (1.9-5.5)	0.7
EPO-receptor density (mean fluorescent intensity)	33 (31-36)	41 (33-47)	0.074	43 (34-45)	34 (30-54)	0.1

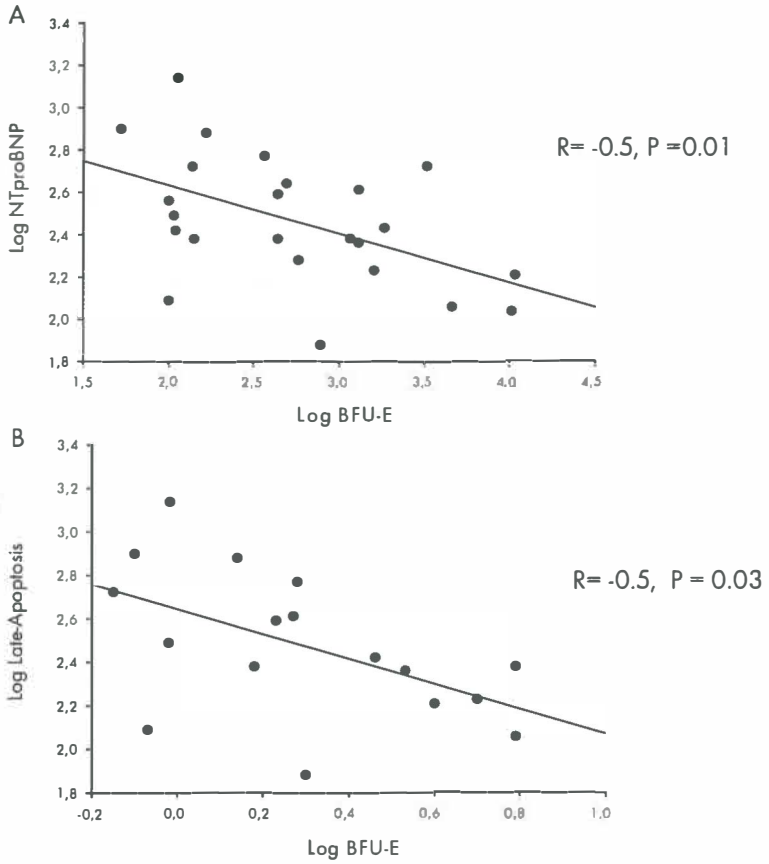
All variables are presented as median + interquartile range. CHF, Chronic Heart Failure; EPO, erythropoietin.

Figure 2. Differences between anemic and non-anemic CHF patients and average Burst Forming Units-Erythroid size.



A. *In vitro* BFU-E formation in anemic and non-anemic CHF patients. **B**. Typical BFU-E colony of a CHF patient and a control subject. **C**. Graphic representation of number of erythroid cells per colony. BFU-E, burst forming unit-erythroid; CHF, chronic heart failure, *; $p<0.05$ versus control.

Figure 3. Predictors of BFU-E formation.



A. Relation beween clonogenic potential and the severity of heart failure. **B.** Relation beween clonogenic potential *in vitro* and bone marrow apoptosis. NTproBNP, N-terminal pro Brian Natriuretic Peptide.

BFU-E formation was identical in anemic and non-anemic CHF patients (202 ± 15 vs 232 ± 35 colonies per 10^4 $CD34^+$ cells and 2 U/mL EPO, P=0.8, figure 2A).The BFU-E colonies of CHF patients appeared smaller and comprised of a lower number of cells (Figure 2B+C).

Predictors of impaired BFU-E formation

A strong inverse correlation was observed between the number of BFU-E-colonies in vitro and the percentage of apoptotic cells in the bone marrow (R=-0.5, P=0.03, figure 3B) or plasma NT-proBNP levels (R=-0.5, P=0.01, figure 3A). When correcting for the potential confounding effects of age, gender, ACE-inhibition and pre-operative hemoglobin levels, NT-proBNP remained an independent predictor of BFU-E formation (B: -0.29 ± 0.09, β: -0.645 partial correlation coefficient -0.6, p<0.01).

Discussion

In the present study we demonstrate a profound bone marrow dysfunction in CHF patients. In a well established *in vitro* erythropoiesis assay, the clonogenic potential of $CD34^+$ HPCs isolated from the bone marrow of CHF patients was at least three-fold lower compared to matched controls. Impaired erythropoiesis *in vitro* was accompanied by a relative depletion of the erythroid lineage and markedly increased apoptosis. Elevated levels of plasma NT-proBNP independently predicted a reduced clonogenic potential, indicating that the extent of erythropoiesis-impairment is related to the severity of heart failure. However, in contrast to our expectations, we did not observe specific EPOR down regulation in erythroid cells suggesting that the sensitivity to EPO is not impaired. Another surprising finding was that the response of erythroid progenitors was comparable in anemic and non-anemic CHF patients. Our findings therefore support the presence of a more generalized dysfunction in CHF, which may increase the susceptibility to anemia. Co-factors including circulating inhibitory factors and increased plasma volume may further explain this process.[6,7]

Several studies have provided circumstantial lines of evidence that suggest the presence of hematopoietic dysfunction in CHF, but withouth the distinction between an intrinsic defect of bone marrow cells versus inhibitory effects of exogenous factors. In the current experiment, we therefore evaluated the cellular function of isolated progenitor cells, which allowed us to exclude potential confounding effects of circulating inhibitory factors or leukocyte mediated suppression of hematopoiesis. Therefore, we were able to establish an intrinsic impairment of erythropoiesis. Reduced erythroid colony formation of isolated $CD34^+$ progenitor cells has so far only been documented in patients with rheumatoid arthritis and idiopathic neutropenia.[11,13] Of note, both these conditions are also associated with an increased incidence of anemia.

Several mechanisms might attribute to hematopoietic dysfunction in CHF. In addition to the reduced number of BFU-Es, the colonies were also smaller with lower number of cells. Furthermore, bone marrow cells of CHF patients displayed markedly increased apoptosis, which inversely correlated with the BFU-E formation. Since EPO regulates physiological apoptosis in erythroid precursors, the observed increased apoptotic cell death during erythropoietic differentiation might explain the reduced clonogenic potential and smaller colony size caused by deficient EPOR signaling. However, in contrast to our expectations, CHF patients did not display down regulation of the EPOR, suggesting unaffected sensitivity to EPO. Moreover, dysfunction of the myeloid and endothelial lineage has been described, suggesting that that the defect is a more generalised phenomenon and might originate in early stem or progenitor cell.[12,14] A specific deficit in EPOR signalling therefore seems unlikely. Enhanced inflammation might also have induced apoptosis and reduced proliferation, which has been demonstrated in mice with heart failure after myocardial infarction.[10] However, by evaluating isolated HPCs we circumvented potential direct effects of inflammatory cytokines or cell mediated cytotoxicity.

Recently, we have demonstrated that circulating leukocytes of CHF patients exhibit accelerated aging, reflected by markedly shorter telomere length.[15] Cells that reach

critically short telomeres either become apoptotic or enter replicative senescence. Telomere length associates with myeloid colony formation of bone marrow MNCs[16] and erythropoiesis,[17] so that the increased apoptosis and the impaired clonogenic potential might thus be explained by replicative exhaustion of HPCs. However, the exact mechanism of HPC-dysfunction remains elusive from our study and warrants further investigation.

Clonogenic potential was similar in anemic and non-anemic CHF-patients, indicating that unexplained anemia in CHF does not merely reflect the severity of bone marrow dysfunction. However, the number of erythroid precursor cells was two-fold lower (P=0.08) in the bone marrow of anemic patients, indicating that despite an equally impaired clonogenic potential, erythropoiesis is further inhibited in anemic patients. Additional erythropoiesis inhibition might be caused by circulating inhibiting factors, which supports our findings that serum of anemic CHF patients inhibits erythropoiesis.[5] The differences between anemic and non-anemic patients should be interpreted with caution because we studied only 6 anemic and 6 non-anemic patients.

To compensate for the bone marrow dysfunction, hematopoietic growth factor levels will increase, which explains, at least in part, the elevated EPO levels in CHF patients. Bone marrow dysfunction will also attenuate the flexibility to adapt erythropoiesis to an increasing demand, augmenting the susceptibility to other causes of anemia. Hence, all conditions that require increased erythropoiesis or that attenuate EPO production might readily result in anemia. In addition to impaired erythropoieis, unexplained anemia in CHF has been associated with an inappropriately low EPO-production by the kidney[6,18], enhanced inflammation[10], circulating hematopoieis inhibitory factors[7] and expansion of the plasma volume, which leads to hemodilution.[6,19] The finding that bone marrow dysfunction alone can not explain anemia is in accordance with the broad range of factors that have been associated with anemia in CHF and might partially explain its multifactorial etiology.[20] In addition to anemia, bone marrow dysfunction might reduce stem cell mediated cardiovascular repair or the efficiency of the immune response, potentially increasing morbidity and mortality even further.

Conclusion

Chronic heart failure causes distinct bone marrow dysfunction which translates into impaired erythropoiesis. Although bone marrow dysfunction warrants patients more susceptible to anemia, it does not exclusively explain its occurrence. Additional research is required to define the type, extent and clinical relevance of bone marrow dysfunction in CHF.

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Chapter 5

Anemia in chronic heart failure: etiology and treatment options

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Abstract

Purpose of the review

Anemia is common in patients with chronic heart failure (CHF), and is related to increased morbidity and motality. The etiology of anemia in heart failure is complex and still not fully resolved. Recombinant human erythropoietin (EPO) might benefit patients with CHF both by correction of anemia and by other extra-hematopoietic effects. This review will describe current advances in the understanding of the pathophysiology of anemia and the potential therapeutic effects of EPO in CHF.

Recent findings

Recent attempts to resolve the preponderant etiology of anemia in CHF have further defined its multifactorial nature. The combination of impaired renal perfusion and function resulting in blunted erythropoietin (EPO) production as well as impaired erythropoiesis in the bone marrow account for a vulnerable erythropoietic system. Moreover, fluid retention has been shown to cause hemodilutional anemia even in the absence of congestive symptoms. The safety and feasibility of EPO to correct anemia in CHF has been established and a recently started phase-3 clinical trial will hopefully provide definitive insight. In addition to the correction of anemia, EPO might improve cardiac function through direct effects on a myocardial EPO receptor and the recruitment of endothelial progenitor cells from the bone marrow. Moreover, EPO exerts important cytoprotective effects during experimental myocardial infarction which is currently evaluated in phase 2 trials. Although EPO shows great promise, we should not neglect other treatable causes of anemia.

Summary

Although the etiology of anemia in CHF is clearly multifactorial, correction of anemia with rhEPO seems promising. In addition to correction of anemia, rhEPO might exert important protective and regenerative properties on the myocardium.

Introduction

Chronic heart failure (CHF) is a final common endpoint of the majority of cardiac conditions, affecting 5 million people in the US and 7 million people in Europe.[1] CHF represents the leading cause of death in the Western world and accounts for an immense health care expenditure, estimated at $ 25 billion dollars in the US annually. The hallmark of the CHF syndrome is left ventricular dysfunction, although the downstream effects of circulatory failure dominate the symptomatic phenotype. CHF is often accompanied by dysfunctions of other organs. In stead of considering these as co morbidities, systemic organ dysfunction is increasingly recognized as an intricate part of the CHF syndrome. For example, the extent of kidney dysfunction more accurately predicts survival in CHF patients than left ventricular ejection fraction, suggesting that the peripheral effects of CHF modulate outcome.[2] Research on the etiology and possible reversal of the peripheral effects of CHF might therefore significantly improve clinical outcome. However, since most of the peripheral CHF syndrome result directly from circulatory failure, reversal seems hard to attain. Anemia in CHF might be an exception since it can be pharmacologically regulated, and has therefore spiked enthusiasm in the heart failure community.[3,4] The present review discusses recent advances in the understanding of the etiology of anemia in CHF and the first results of treatment of CHF patients with erythropoietin stimulating proteins.

The importance of anemia

Anemia, defined by the World health organisation as Hb levels <13 g/dL in men and <12 g/dL in women, is observed in 4 % to 61 % of chronic heart failure (CHF) patients.[4] The prevalence of anemia increases with disease progression, although actual numbers are inconsistent due to the wide range of hemoglobin (HB) cut offs used to define it. Anemia causes chronic volume overload to the left ventricle which results in increased oxygen consumption, left ventricular dilatation and left ventricular hyperthrophy, thereby negatively affecting cardiac function in CHF. Indeed, Post-hoc analysis of multiple randomised controlled trials in CHF patients have consistently and independently associated the presence of anemia with an impaired survival.[4] Recent evidence also indicates that the prognostic significance of anemia can be extended to isolated cohorts of CHF patients with a preserved ejection fraction and non-ischemic etiologies.[5,6] Komajda et al. recently revealed that the new onset of anemia at any time during the 5 year follow up of the COMET trial was independently associated with an impaired survival.[7] These data clearly indicate that the development of anemia should be considered a grave sign in any CHF patient. Moreover, the consistent association between anemia and impaired survival in CHF suggests a potent therapeutic potential for correction.

Etiology of anemia

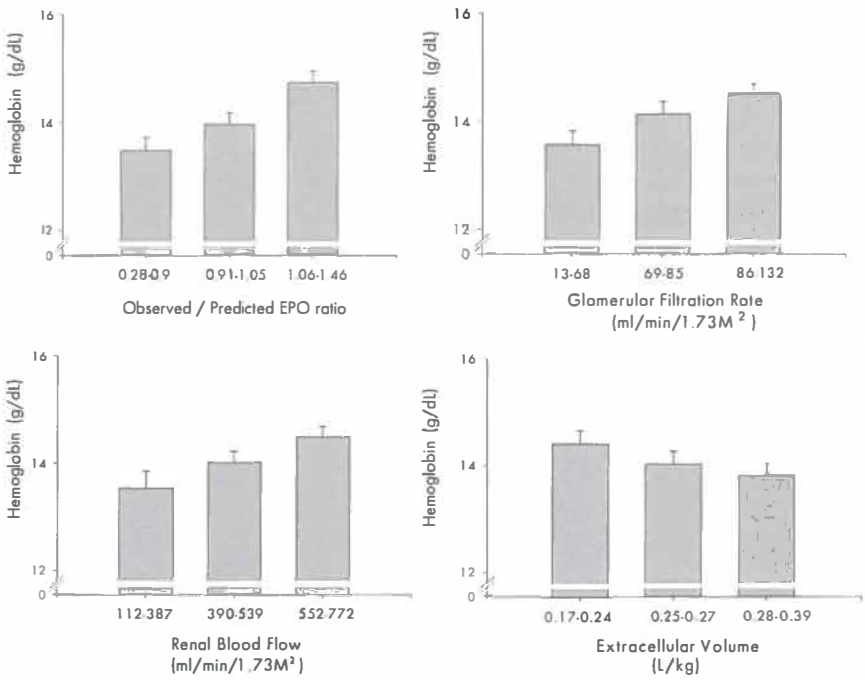
Blunted erythropoietin (EPO) production

Recent studies have importantly improved our understanding of the etiology of anemia in CHF, but it is still far from resolved. The etiology is likely to be multifactorial within the population as well as within each subject.[3,4] Chronic kidney disease often accompanies anemia in CHF but in contrast to these patients, circulating EPO levels are elevated in CHF, increase with the progression of disease, and independently predict impaired survival.[8] However, when EPO levels are corrected for the prevailing Hb by calculating the Observed / Predicted (O/P) EPO ratio, the vast majority of anemic CHF patients display signs of insufficient EPO production. Indeed, Opasich et al. demonstrated that over 90% of anemic CHF patients have significantly depleted O/P EPO ratios compared to healthy controls.[9] Moreover, despite slightly elevated circulating EPO levels, anemic CHF patients also have markedly lower O/P ratios than their non-anemic counterparts.[10] Importantly, these findings are not restricted to patients with coinciding chronic kidney disease, indicating that the blunting of EPO production precedes renal failure. Thus, anemia in CHF is not merely caused by insufficient EPO-production but rather an inability to sufficiently adapt production to an increasing demand. Blunted EPO production is caused by decreased renal perfusion resulting in impaired renal function which both independently predict anemia in CHF. [10] (Figure 1) Furthermore, circulating inflammatory cytokines and ACE-inhibitors can directly inhibit EPO production in the kidney and might also contribute to the blunted EPO production.

Depression of bone marrow function

The relatively elevated EPO levels in non-anemic CHF patients are indicative for a reduced responsiveness of erythropoietic cells to EPO. Indeed, bone marrow of mice with heart failure after myocardial infarction exhibits markedly impaired erythropoiesis and decreased numbers of erythropoietic progenitor cells.[11] IL-1, TNF-α and interferon α, β and γ directly inhibit the formation of mature erythropoietic cells from erythropoietic progenitors in the bone marrow.[12] CHF is frequently associated with elevated levels of these cytokines, and markers for inflammation are independently related to elevated EPO levels.[10,13] Moreover, we have recently demonstrated that anemia in CHF is partially explained by elevated levels of AcSDKP, a negative regulator of hematopoietic stem cells.[8] The inhibitory effects of inflammatory cytokines and AcSDKP will increase the EPO levels required to maintain adequate red blood cell production. Moreover, leukocytes from CHF patients show signs of accelerated aging and the functional capacity of several leucocytic subsets is impaired.[14,15] Since circulating leucocytes share ancestry in the hematopoietic stem cell, the latter might be functionally exhaust. Thus, independent of the presence of anemia, bone marrow function in CHF is depressed through exogenous inhibitory factors and an functional exhaustion of the hematopoietic stem cell pool.

Figure 1. Relation between hemoglobin levels and erythropoietin, renal perfusion.



Glomerular Filtration Rate and extracellular volume. EPO; erythropoietin, ERPF; effective renal plasma flow, GFR; glomerular filtration rate, ECV; extracellular volume.

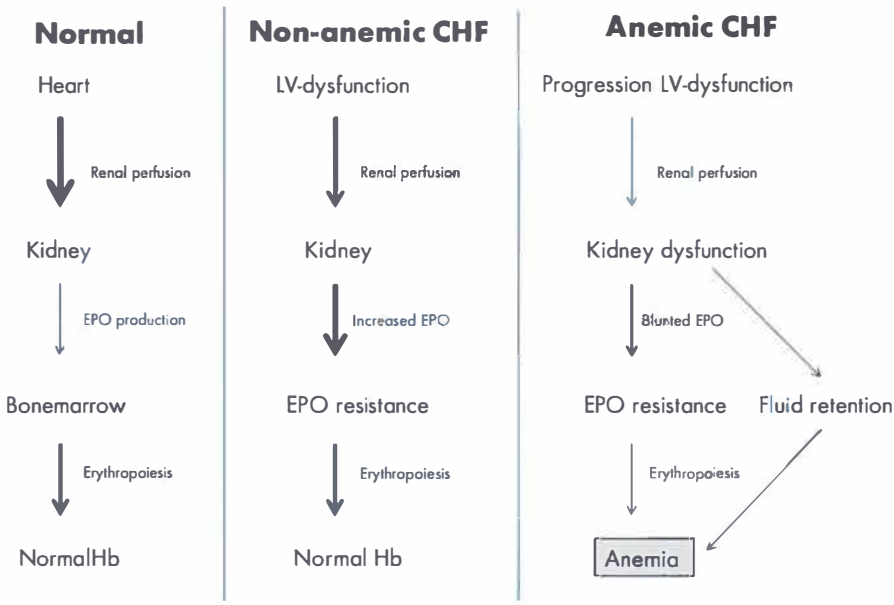
Congestion

Fluid retention due to impaired renal perfusion in CHF can result in expansion of plasma volume and consequent hemodilution which may cause pseudo-anemia. Until recently pseudo-anemia was considered to be restricted to patients with acutely decompensated or end stage heart failure.[16] Recently we determined the extra cellular volume of 97 stable CHF patients on outpatient follow up at our department with a novel radionuclotide measurement. Anemic subjects displayed significantly higher extracellular volume and fluid retention was an independent predictor of anemia. No differences in signs and symptoms for congestion were observed between anemic and non-anemic subjects, indicating that subclinical fluid retention can already result in anemia.[10] Therefore, in addition to blunted EPO production and attenuated erythropoietic capacity of the bone marrow, subclinical fluid retention seems an important cause of anemia in CHF. (Figure 1)

Iron deficiency

CHF is infrequently associated with standard biochemical indices of haematinic deficiencies. Nanas et al. however recently reported significantly depleted iron stores

Figure 2. Possible causes contributing to the development of anemia in CHF.



in the bone marrow aspirates of 27 out of 37 anemic patients with end stage heart failure.[17] Although these results are indicative for the presence of reduced iron supplies in the bone marrow, and therefore reduced iron supply for erythropoiesis, a non-anemic control group was unfortunately not included. Diversion of iron into the reticuloendothelial system is intrinsic to chronic disease states and iron deficiency is almost equally common in non-anemic CHF patients.[10,12] Therefore, the presence of reduced iron supplies in the bone marrow does not necessarily explain the occurrence of anemia. Prospective controlled studies are required to fully elucidate the role of iron deficiency in CHF.

In summary, recent studies that aimed to identify the preponderant etiology of anemia in CHF have further defined its multifactorial nature. Many factors associated with anemia in CHF are also prevalent in non-anemic subjects. Bone marrow of CHF patients requires higher EPO levels to maintain adequate erythropoiesis due in part to functional exhaustion of stem cells as well as inhibition by inflammatory cytokines and ACE-inhibitors. Concomitantly, iron supplies are diverted from the bone marrow, further restricting erythropoiesis. Together these factors account for a vulnerable erythropoietic system. With disease progression, further deterioration of renal perfusion will cause kidney dysfunction and blunt EPO production, impairing the ability to respond to an increasing demand. Moreover, the impaired renal hemodynamics stimulate fluid retention and plasma expansion causing hemodillutional anemia. (Figure 2)

Recombinant human erythropoietin

The observation that anemia in CHF is associated with relatively insufficient EPO production and increased EPO-requirements in the bone marrow has prompted the evaluation of rhEPO for it's treatment. After an initial uncontrolled study the Israeli group of Silverberg et al. randomised 32 patients with mild anemia to receive either rhEpo and IV iron or no additional treatment, resulting in significant improvement in New York Heart Association (NYHA) functional class left ventricular ejection fraction, renal function and heart failure hospitalizations.[18] These findings have been corroborated by other open label studies, causing great enthusiasm in the cardiovascular community.[19,20] Based on these findings, 3 double blind placebo controlled phase 2 clinical studies were designed to evaluate the effect of 26 weeks treatment with the rhEPO analogue darbepoetin alfa (AMGEN, CA, USA) on exercise capacity, NYHA functional class, left ventricular ejection fraction and quality of life.[21,22] Although EPO treatment with a fixed dose or a weight based approach successfully restored Hb levels to normal levels, the effects of EPO on NYHA class, aerobic capacity, LVEF, and heart failure hospitalisations could not be confirmed. Since the AMGEN trials were properly designed, the results of these smaller open label studies might have arisen from chance or their open label design. The exercise tolerance and quality of life however showed favourable trends towards improvement, partially attaining statistical significance. Moreover, a pooled analysis of the two largest studies, which combined the data of more than 480 patients, revealed a trend ($p=0.074$) to reduced risk of the combined endpoint of CHF-related hospitalization and all-cause mortality in the rhEPO treated groups.[23] Nevertheless, these studies were not powered for analysis of survival and the conclusions drawn are tantalizing but premature. A large multicenter, double-blind, randomized, placebo-controlled trial has recently started enrolling patients (RED-HF) will hopefully provide a definitive answer.

Safety issues

In the fall of 2006 the CHOIR and CREATE studies were published , demonstrating that normalization of Hb values to reference ranges (Hb > 13.5 g/dL) with rhEPO in chronic kidney disease patients resulted in significantly more cardiovascular events compared to target levels of 11.3 g/dL.[24,25] Moreover, several trials in oncology patients have been prematurely discontinued because of increased mortality in the EPO treated groups.[26] As a result, the FDA has recently issued a black box warning for erythropoiesis stimulating agents recommending "the lowest possible dose to slowly raise the hemoglobin concentration to the lowest level that will avoid the need for a blood transfusion." The multicenter, double-blind randomised controled Trial to Reduce Cardiovascular Events with Aranesp Therapy (TREAT) which evaluates the effect of darbepoetin alfa on cardiovascular events in CKD patients, has recently been evaluated by the data safety monitoring committee which saw:"no cogent reasons to recommend alteration or termination of the trial". (personal communication) In contrast to the studies described above, the TREAT study is blinded and adequately powered to evaluate mortality, indicating that the recent scrutiny might be premature.

Of note, the etiology of anemia in CHF seems distinct from patients with chronic kidney disease and oncology. Hence, extrapolation of the results from these trials is thorny. Moreover, no deleterious effects of rhEPO have been observed in CHF patients so far, but current trials will be carefully monitored.

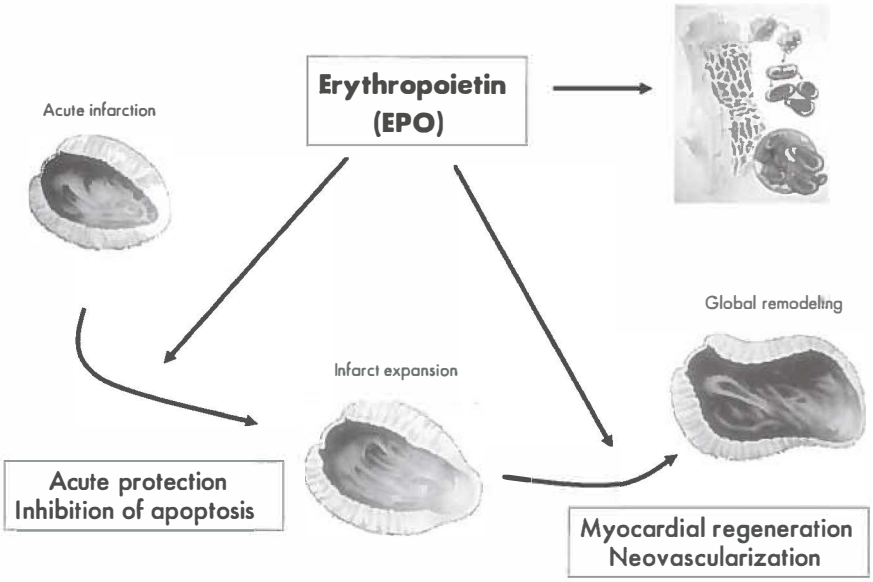
Extra-hematopoietic effects of EPO

The principal effect of EPO in the bone marrow is the reduction of the physiological apoptosis associated with cell turnover in erythroid progenitor cells. However, in conjunction with other growth factors EPO also stimulates proliferation and differentiation of these cells.[27] Recently, these properties of EPO have been extended to the heart. Following an acute myocardial infarction, extensive myocardial apoptosis ensues which in part determines the extent of the permanent myocardial damage. EPO exerts potent anti-apoptotic effects on the myocardium and numerous studies have translated the cytoprotective in vitro effects into ex and in-vivo models of acute myocardial infarction in rodents, rabbits and dogs.[28] We recently performed a randomised safety and feasibility study with a single bolus of EPO, administered during primary PCI for a first acute MI. EPO was both safe and well tolerated, caused only a small but non-significant increase in hematocrit levels and significantly increased circulating endothelial progenitor cells.[29] These findings led to the design of a randomised multicenter study that evaluates whether EPO can attenuate post-MI loss of cardiac function, currently enrolling patients (NCT00449488). The similar REVEAL trial is currently performed in the US (NCT00378352). In addition, EPO has consistently been shown to improve cardiac function in experimental models of chronic myocardial dysfunction.[30-35] The improvement of cardiac function is consistently associated with restoration of microvascular dysfunction, mediated through a combination of endothelial progenitor cell and vascular endothelial growth factor mediated neovascularization.[36] The beneficial effects of EPO in experimental CHF can also be induced with doses that do not affect hematocrit levels.[37] These significant extra hematopoietic effects suggest that EPO might also benefit non-anemic patients with cardiac disease. Furthermore, depending on the time after an ischemic insult, EPO exerts cytoprotective effects and in a later stage facilitates regeneration of the myocardium indicating a broad therapeutic window. (figure 3)

Other treatment options

Since the etiology of anemia in CHF seems to result form a combination of predisposing factors, correction of any of these components might also resolve anemia. Bolger et al. recently evaluated the effect of intra venous administration of iron sulphate alone on hemoglobin levels in 17 anemic CHF patients.[38] They observed a significant increase in hemoglobin levels suggesting that intra venous iron might be used to treat all anemic CHF patients. The increase in hemoglobin levels in the total population was however driven by patients that were actually iron deficient. Despite the lack of a proper control group, the study clearly indicates that any of the classical causes of anemia

Figure 3. Therapeutic potential of erythropoietin after myocardial infarction.



should first be treated before starting with more sophisticated tools. Because CHF is not associated with iron deficiency but rather a decreased iron supply for erythropoiesis, unrestricted supplementation of iron might result in toxic accumulation of iron in the reticuloendothelial system.[39] Fortunately, the IRON-HF study will evaluate the use of intravenous iron CHF in a controlled fashion.[40] Finally the fact that anemia in CHF is in part mediated through hemodilution, in spite of congestive symptoms, suggest that intensification of the diuretic regimen might be sufficient to raise hemoglobin levels. Hemodilutional anemia should indeed be considered in all anemic CHF patients, but uncontrolled administration of high dose diuretics might further impair kidney function in euvolemic patients. Since congestive signs and symptoms poorly predict actual volume status, radionucleotide based measurements should be used to identify patients with hemodillutional anemia.

Conclusions

Anemia has been recognized as a component of the peripheral CHF syndrome, which importantly modulates mortality. Recent studies that aimed to identify the etiology of anemia in CHF have further defined its multifactorial nature. Although correction of anemia with rhEPO has recently been scrutinized for other indications, in CHF it so far seems safe and well tolerated. In addition to correction of anemia, rhEPO might exert important protective and regenerative properties on the myocardium.

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PART 2

Mechanisms of erythropoietin induced improvement of cardiac function

Chapter 6

Erythropoietin improves cardiac function through endothelial progenitor cell and vascular endothelial growth factor mediated neovascularization

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Abstract

Aims

Erythropoietin (EPO) improves cardiac function and induces neovascularization in chronic heart failure (CHF), although the exact mechanism has not been elucidated. We studied the effects of EPO on homing and incorporation of endothelial progenitor cells (EPC) into the myocardial microvasculature and myocardial expression of angiogenic factors.

Methods and results

CHF was induced in rats by coronary artery ligation resulting in myocardial infarction (MI) after bone marrow had been replaced by human placental alkaline phosphatase (hPAP) transgenic cells. We studied the effects of darbepoetin alfa treatment (EPO, 40 μg/kg, every 3 weeks, starting 3 weeks after MI) on longitudinal changes in left ventricular (LV) function, circulating EPC, myocardial histology, and expression of vascular endothelial growth factor (VEGF) determined 9 weeks after MI. EPO prevented LV-dilatation and improved cardiac function (all $P<0.05$), which was associated with 42% increased capillary growth ($P<0.01$). EPO-induced mobilization of EPC from the bone marrow ($P<0.01$), which resulted in a 3-fold increased homing of EPC into the cardiac microvasculature. The percentage of the endothelium that consisted of bone marrow derived cells was significantly increased (3.9 ± 0.5 vs. $11.4 \pm 1\%$, $P<0.001$) comprising 30% of the newly formed capillaries. In addition, EPO treatment resulted in a 4.5-fold increased myocardial expression of VEGF, which correlated strongly with neovascularization ($r=0.67$; $P<0.001$). VEGF was equally expressed by endothelial cells of myocardial and bone marrow origin.

Conclusions

EPO-induced neovascularization in post-MI heart failure is mediated through a combination of EPC recruitment from the bone marrow and increased myocardial expression of VEGF.

Introduction

Chronic heart failure (CHF) represents a complex of symptoms related to impaired cardiac function affecting 5 million people in the US. Despite optimal treatment with current strategies, morbidity and mortality remain high.[1] CHF is associated with myocyte hypertrophy and impaired microvascularization of the myocardium, leading to a mismatch between oxygen demand and supply.[2,3] Current therapies aimed at improving the microvasculature are under investigation, of which erythropoietin (EPO) is one of the most promising therapies.[4–6]

EPO is an erythropoietic growth factor, promoting survival, proliferation, and differentiation of erythroid progenitor cells.[7] We and others have recently demonstrated that EPO-treatment improved cardiac function in experimental models of CHF.[8–12] The improvement of cardiac function is consistently associated with an increase in capillary formation, although the mechanism of neovascularization is unknown.

It has repeatedly been shown that EPO promotes proliferation and survival of endothelial cells in vitro and stimulates angiogenesis in vivo.[13–15] In addition, EPO induces the proliferation, differentiation, and adhesion of a subset of bone-marrow-derived progenitor cells with an endothelial phenotype [endothelial progenitor cells (EPC)] in vitro and results in marked mobilization of EPC in vivo.[8,16–18] EPC specifically home to sites of neovascularization and incorporate into newly formed vessels.[19] It is, however, unknown whether the mobilized EPC contribute to EPO-induced neovascularization.

We hypothesized that EPO-induced neovascularization is mediated through EPC recruited from the bone marrow in addition to in situ proliferation of myocardial endothelial cells. In order to study the effects of EPO on differentiation of EPC into the myocardial vasculature, bone marrow of rats was replaced with labelled bone marrow cells.

Methods

Animals

We used male Fischer F344 rats ($n = 65$) weighing 200–230 g (Charles Rivers, France) as recipients and R26-hPAP rats [$n = 10$, F344 background, ubiquitously expressing human placental alkaline phosphatase (hPAP)] as donors.[20] Animals were fed and housed, according to institutional rules, and the experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Bone marrow labelling

In order to evaluate the effects of EPO on homing and incorporation of EPC into the endothelium, we replaced the bone marrow by genetically labelled bone marrow cells as described in detail previously.[21] Briefly, 24 h after 9 Gray total body irradiation

of recipient rats, whole bone marrow cells (25 x 106 nucleated cells) of donor rats were transfused to recipients. Rats were housed in filtertop cages and drinking water was supplemented with neomycin (0.35% wt/vol), 2 weeks post and prior to irradiation. After 6 weeks, hPAP expression on leucocytes was evaluated by FACS, and rats with chimerism >80% were randomly allocated to MI or sham surgery. Successful haematopoietic recovery was further confirmed by full blood counts and chimerism was re-evaluated at sacrifice. This model allowed us to evaluate differentiation of bone marrow-derived cells into an endothelial phenotype.

Experimental protocol

Rats were randomly subjected to induction of myocardial infarction (MI) or sham surgery as described previously in detail.[11] Briefly, animals were intubated and mechanically ventilated with 2.5% isoflurane in room air enriched with 1.0 L/min oxygen. After left-side thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery. In sham operated rats, the same surgery was performed without ligating the suture. Three weeks after coronary artery ligation, rats with MI were subjected to treatment with the long-acting EPO analogue darbepoetin alfa (40 µg/kg, Aranesp, Amgen Inc., Thousands Oaks, CA, USA) or saline, administered intraperitoneally once every 3 weeks. Treatment allocation was balanced for left ventricular (LV) end-diastolic diameter and LV fractional shortening determined by echocardiography. The dose of darbepoetin was based on our previous experiments, demonstrating increased neovascularization in this model.[11] At baseline, at week 3 (before therapy) and weeks 6 and 8, cardiac function was determined by echocardiography. After 9 weeks, haemodynamic function was assessed invasively, thereafter hearts were rapidly excised and weighed. Myocardial tissue was transected transversally and processed for immunohistochemistry or snap frozen for western blot analysis.

Echocardiographic measurements

Cardiac function was prospectively assessed by echocardiography (Vivid 7, GE Healthcare, Chalfont St Giles, UK; equipped with a 10-MHz phase array linear transducer). The echocardiographic measurements were performed under general anesthesia with 2.5% isoflurane, by two researchers blinded for the treatment allocation. Both 2-dimensional (2D) images in parasternal long-axis and short-axis view and 2-D guided M-mode tracing were obtained. Long-axis views were obtained, ensuring that the mitral and aortic valves and the apex were visualized. Short-axis views were recorded at the level of mid-papillary muscles. LV end-systolic diameter (LVESD) and LV enddiastolic diameter (LVEDD) were measured from the M-mode and calculated as an average from short- and long-axis view. LV fractional shortening (FS %) was calculated as FS = (LVEDD - LVESD)/LVEDD x 100%. LV ejection fraction (EF %) was alculated using the Teichholz method of estimated LV volumes.[12]

Hemodynamic measurements

At sacrifice, rats were anesthetized and a microtip pressure transducer (Millar Instr. Inc., Houston, TX, USA) was inserted into the LV cavity via the right carotid artery.

After a 3-min period of stabilization, heart rate (HR), LV systolic pressure (LVSP), LV end-diastolic pressure (LVEDP), and developed LV pressure (dLVP = LVSP - LVEDP) were measured. As indices of contractility and relaxation, the maximal rates of increase and decrease in LVP (dP/dtmax and dP/dtmin) were determined. The catheter was retracted into the aortic arch and arterial systolic/diastolic blood pressures (SBP, DBP) were recorded.

Infarct size, myocyte hypertrophy, and capillary density

Infarct size, myocyte hypertrophy, and capillary density were determined as described in detail previously.[11] Briefly, infarct size was determined by planimeter in transverse slices on picrosirius red/fast green–stained sections and expressed as the percentage of scar length to total LV circumference. Concentric myocyte hypertrophy, in the viable LV wall remote from the infarct, was measured in deparaffinized sections stained with Gomori's silver staining, as the cross-sectional area of transversally cut myocytes showing a nucleus, and averaged per tissue area. Endothelial cells were stained with biotin-labelled GSL-Lectin (1:100; Sigma-Aldrich, St Louis, MO, USA), a size criterion of 10 μm was used to exclude small arterioles and venules, and image analysis was used to measure capillary density in the viable LV-wall, calculated as the number of capillaries per tissue area in the all transverse slice. As a measure of neovascularization, capillary-to-myocyte ratio was calculated dividing capillary with myocyte density.

Circulating endothelial progenitor cells

Whole blood was collected in heparine tubes (17 IU/mL). Mononuclear cells were isolated using Histopaque-1083 (Sigma Chemical, St Louis, MO, USA) according to the instructions supplied by the manufacturer. Isolated mononuclear cells (1 x 106) were seeded on fibronectin-precoated 24-well plates (BD BioCoat, Bedford, MA, USA) in EndoCult medium (StemCell Technologies, London, UK) supplemented with Penicillin (100 U/mL) and Streptomycin (100 μg/mL). After 6 days, adherent cells were washed with medium, incubated with 1,1'-dioctadecyl-3,3,3', 3'-tetramethylindocarbocyanine-labelled acetylated LDL (10 μg/mL Dil AcLDL; MolecularProbes, Invitrogen, Carlsbad, CA, USA) for 12 h, fixed with 1% paraformaldehyde for 10 min, and counterstained with fluorescein isothyiocyanate-labelled Griffonia (bandeiraea) simplicifolia lectin I, isolectin B4 (lectin, 10 μg/mL; Vector Laboratories, Burlingame, CA, USA) and DAPI for nuclear staining. Images were captured by an LSM 410 confocal microscope (Carl Zeiss, Jena, Germany). Cells double positive for DiI AcLDL and lectin staining were considered EPC and counted in high-power fields by co-localization analysis (Image-Pro Plus for Windows, version 4.5.0.29). For every rat, cells were seeded in duplicate and an average number of EPC was calculated per well from five high-power fields.

Fluorescent microscopy

Cryosections were stained with primary antibodies [rabbit antihPAP, serotec, London, U.K., mouse anti-rat His52 (rat endothelial cell antigen) a kind gift from

Dr J.L. Hillebrands, and mouseanti-vascular endothelial growth factor (VEGF), C-1, Santa Cruz] followed by FITC labelled goat anti rabbit-IgG- and TRITC, Alexa 555 or Cy5-labelled goat anti-mouse-IgG-isotype specific secondary antibodies. For nuclear staining, sections were mounted in vectashield mounting medium containing DAPI. Analysis was restricted to the viable LV wall. Cells that stained positive for hPAP were considered bone-marrow-derived and cells double positive for hPAP and His-52 were considered bone-marrow-derived endothelial cells. Cells were enumerated per high power field by two researchers (B.D.W., H.O.) blinded for the treatment allocation in five randomly chosen high power fields of the non-infarcted LV wall. The percentage of the endothelium composed of bone-marrow-derived cells was calculated by co-localization analysis. The surface area that stained double positive for hPAP and his52 is expressed as percentage of the total his-52 positive area.

Erythropoietin, erythropoietin-receptor, and vascular endothelial growth factor expression

In order to evaluate the effects of EPO on expression of angiogenetic factors and the EPO receptor, samples of the viable LV free wall (non-infarcted area) were snap frozen in liquid nitrogen and stored at -80°C. The expression of EPO, EPO-receptor, and VEGF was determined in tissue homogenates of seven randomly selected rats per treatment group by standard western blotting techniques under denaturing conditions, as described previously.[23] Membranes were reprobed for GAPDH to confirm equal protein loading and transfer. Signals were detected by the ECL-detection method and quantified by densitometry. Results are expressed as arbitrary units and represent the ratio between EPO, EPO-receptor, or VEGF and GAPDH per lane. Primary antibodies were purchased from Santa Cruz biotechnology [EPO H126, 1:250; EPO-receptor M-20, 1:500; VEGF C1, 1:250 (recognizes all splice variants of VEGF)] or Fitzgerald Industries (GAPDH 6c5, 1:10000) and horeseradish peroxidase conjugated anti-mouse or anti-rabbit IgG were used as secondary antibodies.

Plasma erythropoietin and vascular endothelial growth factor levels

Plasma levels of EPO and VEGF levels were determined by a commercial Enzyme Linked Immunosorbent Assay according to the guidelines provided by the manufacturer (Quantikine, R&D systems, Londen, UK).

Statistical analysis

Data are presented as mean ± SEM when normally distributed and median + interquartile range when skewed distributed. Differences among groups were tested using one-way analysis of variance, followed by LSD post-hoc analysis if normally distributed, and by Kruskal–Wallis test followed by Mann Whitney U test with Bonferroni correction when skewed. Correlations were assessed with Spearman's correlation test. All reported probability values are twotailed, and a P-value ,0.05 was considered statistically significant. All statistical analyses were performed with SPSS version 12.0.

Table 1. General characteristics and myocardial function.

	Sham	MI	MI-EPO
General			
n	9	12	12
Infarct size (% of LV)#	-	43±1	41±2
Hemodynamics			
Heart rate (bpm)	300±17	274±11	295±8
LVSP (mmHg)	125±6	108±4†	120±3‡
LVEDP (mmHg)	13±3	22±1†	16±1‡
dLVP (mmHg)	112±8	86±5†	103±4‡
SBP (mmHg)	114±14	108±4	120±3
DBP (mmHg)	88±3	78±4	90±2§
dP/dT max (mmHg/sec)	11847±761	9180±537†	10896±336*‡
dP/dT min (mmHg/sec)	-10368±800	-7311±595†	-8749 ±287*‡
Body/organ weight			
BW (g)	293±5	281±5	290±6
Heart weight/BW(mg/g)	3.0±0.9	4.0±0.7†	3.6±0.2†‡

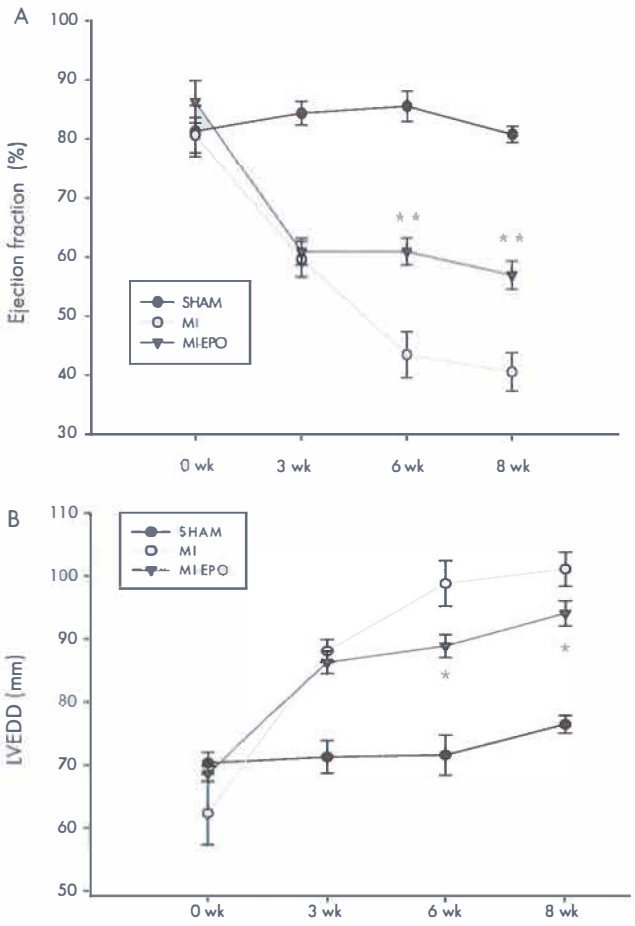
Data are presented as mean ± SEM; n indicates number of animals; bpm, beats per minute; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; dLVP, developed left ventricular pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; dP/Dt max and dP/dT min, maximal rates of increase and decrease in LVP; BW, bodyweight. # Infarct size, as % of LV-circumference of the mid papillary slice. *p<0.05; †p<0.01 vs. Sham; ‡p<0.05, §p<0.01 vs. MI.

Results

Erythropoietin prevents progression of left-ventricular-dysfunction and improves capillary density, associated with marked mobilization of endothelial progenitor cells

Three rats were excluded from analysis due to failed bone marrow transplantation. Twenty-four hour mortality after MI was 31% (16 rats) and three rats (two in MI–EPO and one in MI) were excluded because the infarct size was <25% of the LV circumference. The final population comprised 12 rats per MI group and 9 rats in the sham group. General characteristics are presented in Table 1. Although hematocrit values were comparable between groups at the start of treatment, EPO treatment resulted in maximal 31% increase in hematocrit increasing from 50 ± 0.3 to 64 ± 1% 1 week after treatment. Although the hematocrit decreased during the following 2 weeks until the next EPO treatment, it remained significantly elevated in MI–EPO compared with sham and MI throughout the experiment (all $P < 0.01$). Serial echocardiographic parameters are presented in Figure 1. Induction of MI resulted in marked enlargement of LVEDD and deterioration of LV performance (EF) at week 3.

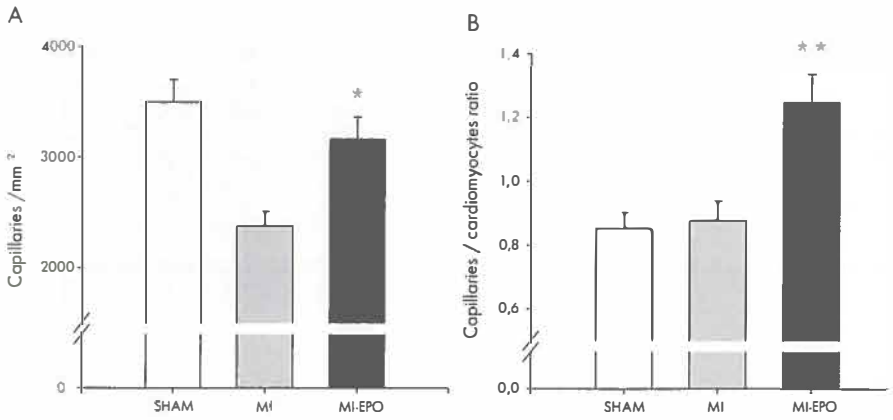
Figure 1. Effect of EPO on myocardial remodeling after myocardial infarction.



Changes in echocardio-graphic indices of LV function and LV end diostolic diameter during 8-weeks follow-up after coronary artery ligation.
* p<0.05 vs. MI, ** p<0.01 vs. MI. LVEDD indicates left ventricular end-diastolic diameter

In the following weeks, the deterioration of LV performance and LV dilatation (LVEDD) progressed in the untreated MI group, but remained stable in the EPO treated group (all P<0.05). LV-systolic pressure, LV developed pressure, myocardial contractility (dP/dtmax) and relaxation (dP/dtmin) and LVEDP were all significantly impaired in both MI groups compared with sham, and significantly improved after EPO treatment (Table 1). Histological infarct size was comparable between all MI groups. Cardiomyocytes' crosssectional area increased in both MI groups compared with sham (P<0.01), but was not significantly different between MI and MI–EPO.

Figure 2. Effect of EPO treatment on neovascularization.



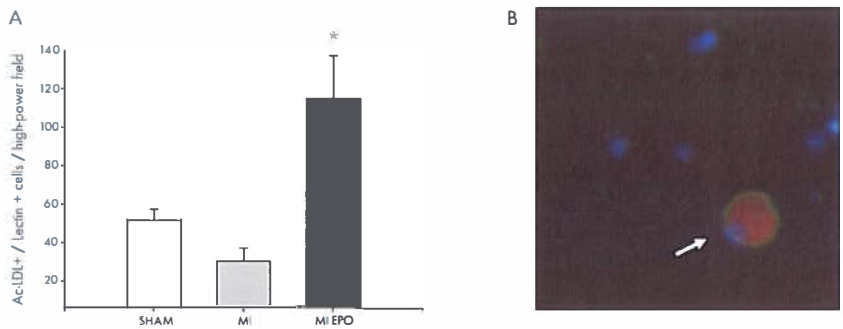
A. Measurements of capillary density in number of capillaries per mm2. **B.** Bar graph representing the capillary-to-myocyte ratio in different groups. *p<0.05 vs. MI, ** p<0.01 vs. MI

Capillary density was significantly reduced in the saline treated MI group ($P<0.01$). EPO treatment increased capillary density by 33% (Figure 2, $P<0.01$) restoring it to sham levels. The capillary-to-myocyte ratio increased by 42% in the EPO treated group compared with sham and MI (Figure 2, $P<0.01$), indicating the formation of new vessels, rather than resulting from reduced cardiomyocyte hypertrophy. Treatment with EPO resulted in a four-fold increase in the number of circulating EPC compared with the MI group ($P<0.01$; Figure 3).

Erythropoietin augments homing and incorporation of endothelial progenitor cells into the myocardial microvasculature

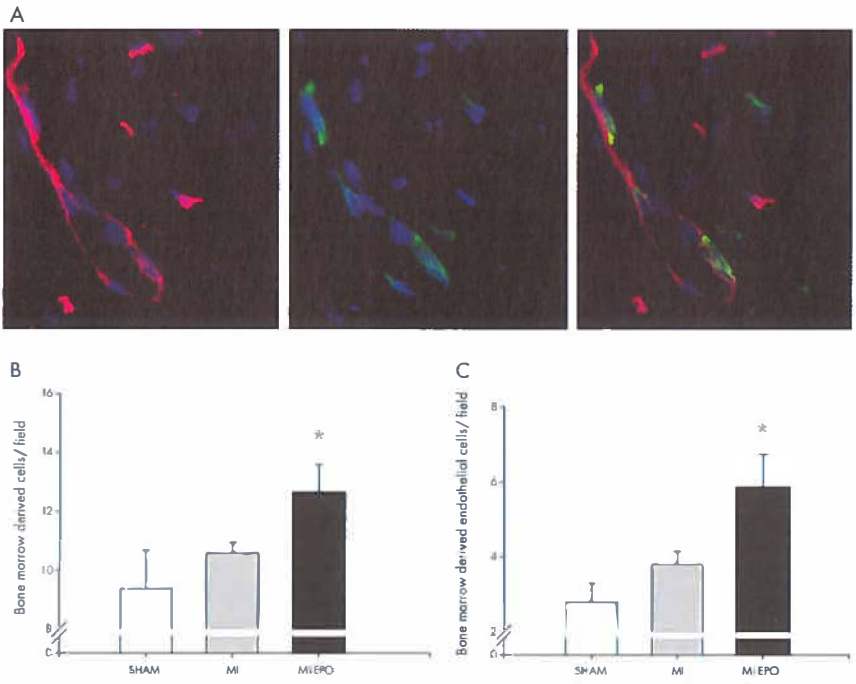
In the non-infarcted LV free wall of the MI rats, a slight nonsignificant increase in the number of bone-marrow-derived cells (hPAP+) and bone-marrow-derived endothelial cells (hPAP+, his 52+) was observed compared with sham (Figure 4). This was markedly stimulated by EPO treatment, resulting in a 29% increased influx of bone marrow- derived cells and a 2.1-fold increase in bone-marrow-derived endothelial cells ($P<0.05$) The percentage of the endothelium that comprised bone-marrow-derived cells did not differ significantly between MI and sham groups. However, this was markedly augmented by EPO treatment, increasing from 3.9 $\pm$ 0.5 to 11.4 $\pm$ 1% (Figure 4; $P<0.001$ vs. sham and MI). EPO treatment resulted in a 33% increase in capillary density and the percentage of the endothelium that comprised bone-marrow-derived cells increased from 4 to 11%. Therefore, ~30% of the newly formed endothelium comprised bone marrow-derived cells. The enhanced incorporation resulted from specific homing of EPC, reflected by a significantly higher ratio between bone marrow-derived endothelial cells and total bone marrow-derived cells ($P<0.001$, Figure 4).

Figure 3. Effects of EPO treatment on number of circulating Endothelial Progenitor Cell.



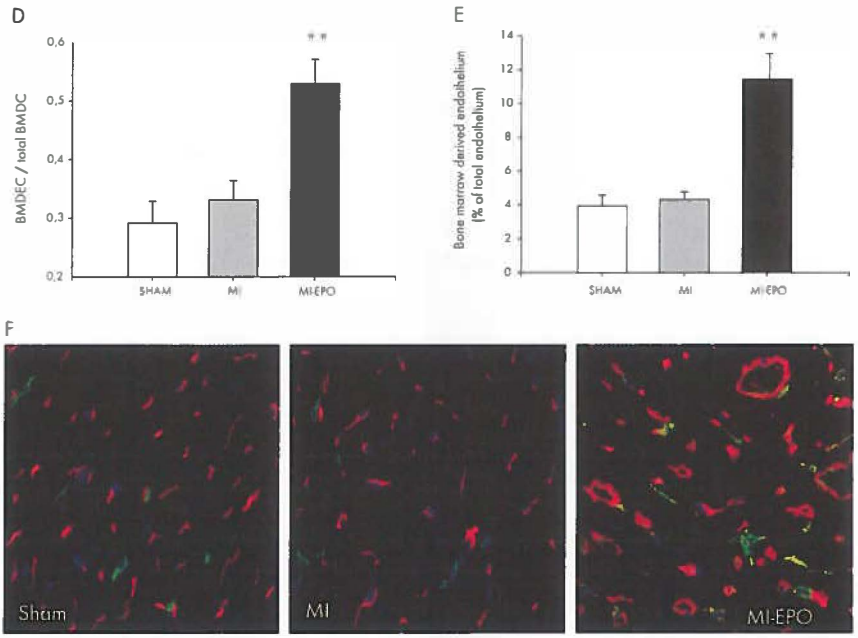
A. Graphic representation of number of EPC. **B**. Endothelial progenitor cell under high magnification (white arrow), positively stained for DiI AcLDL (red cytoplasm) and lectin (green cytoplasm), including DAPI nuclear staining (blue). * p<0.01 vs. MI.

Figure 4. Effect of EPO treatment on incorporation of endothelial progenitor cells into the myocardial vasculature.



A. Myocardial section stained with hPAP bone marrow derived (green), His 52 (endothelium, red) and DAPI (nucleus, blue) detected at 63% magnification. The three panels display the endothelium, bone marrow derived cells and the fluorescent overlay respectively, demonstrating bone marrow derived endothelial cells (BMDEC) which appear yellow. **B**. Bar graph representing the total number of bone marrow derived cells in the myocardium. **C**. Bar graph representing the numbers of BMDEC per high power field.

Figure 4. Continued.



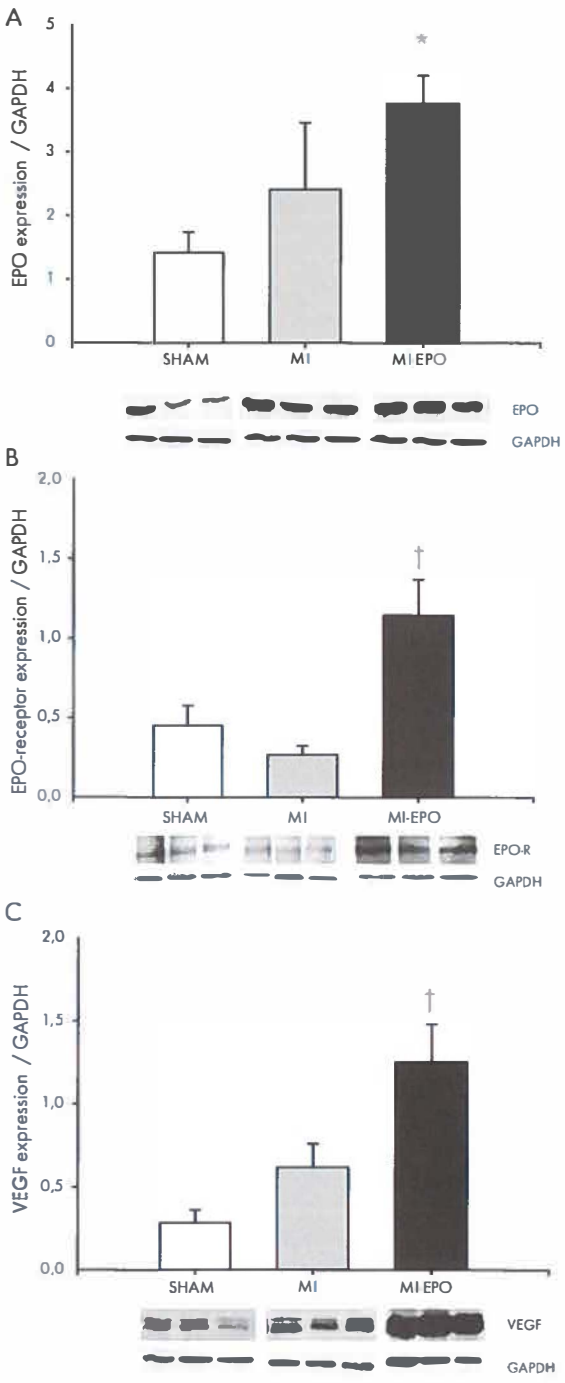
D. Bar graph representing the ratio between BMDEC and total bone marrow derived cells. **E**. Bar graph representing the percentage of endothelium comprised of bone marrow derived cells. **F**. Representative fluorescent overlay of the treatment groups at 40x magnification, BMDEC appear yellow. BMDC, bone marrow derived cells; BMDEC, bone marrow derived endothelial cells; hPAP, human placental alkaline phosphatase. *p<0.05 vs. MI, ** p<0.01 vs. MI.

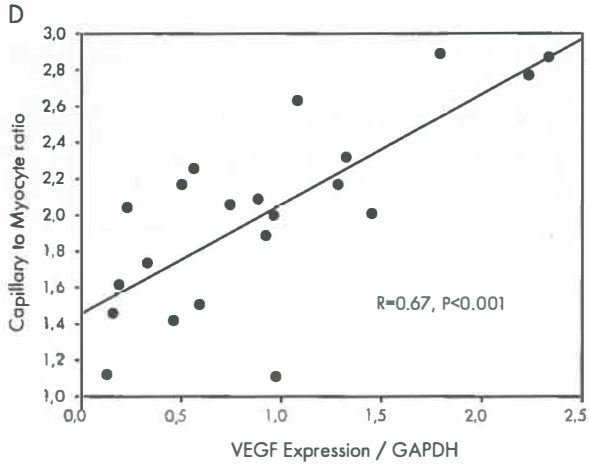
Moreover, a strong correlation was observed between circulating EPC and myocardial bone-marrow-derived endothelial cells ($R = 0.64$, $P < 0.001$) indicating that the increased EPC incorporation was directly related to increased EPC mobilization.

Erythropoietin induces vascular endothelial growth factor expression and upregulates its own receptor

Three weeks after the last EPO administration, plasma EPO levels were comparable between MI groups [20 (2–76) vs. 18 (1–29) pg/mL in MI and MI–EPO groups, respectively], and slightly but non-significantly higher than sham [7 (1–30) pg/mL]. Myocardial expression of EPO was however two-fold higher in both MI groups ($P < 0.05$, Figure 5). EPO-receptor expression was slightly decreased in MI compared with sham group, EPO treatment resulted in a 3-fold upregulation of EPO-receptor expression ($P < 0.01$ vs. sham and MI, Figure 5). Although plasma VEGF levels were comparable among groups [7 (2–69), 12 (1–39), 8(1–29) pg/mL in sham, MI, MI–EPO groups,

Figure 5. Expression of EPO, EPO-receptor and VEGF in the left ventricular free wall.





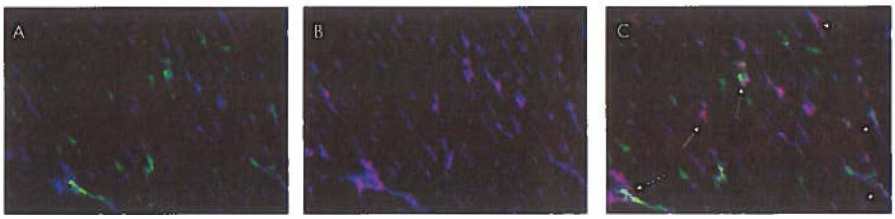
A. Bar graph and representative bands showing the difference in the expression of EPO in the left ventricular free wall. **B**. Bar graph and representative bands showing the difference in the expression of EPO-receptor in the left ventricular free wall. **C**. Bar graph and representative bands showing the difference in the expression of VEGF in the left ventricular free wall. **D**. Scatter plot delineating the relation between capillary to myocyte ratio and VEGF expression in the left ventricular free wall of all groups (sham, MI and MI-EPO). EPOR, EPO-receptor; VEGF; vascular endothelial growth factor. *p<0.05 vs. MI, † p<0.01 vs. MI

respectively], a moderate increase in the expression of VEGF was observed in the MI-group (Figure 5, P = 0.07 trend), but expression of VEGF was increased 4.5-fold after EPO treatment (P < 0.01 vs. sham P < 0.02 vs. MI). The expression of VEGF was strongly correlated with new capillary growth (Figure 5, R = 0.67, P = 0.001). Triple staining with hPAP, His-52, and VEGF antibodies revealed that VEGF immunoreactivity was equally expressed in the endothelial cells of bone marrow and myocardial origin (Figure 6).

Discussion

The present study demonstrates for the first time that bone-marrow-derived progenitor cells are involved as a cardiac repair mechanism of systemic EPO treatment. EPO induced neovascularization is associated with increased mobilization, myocardial homing, and vascular incorporation of EPC, which comprise 30% of the newly formed endothelium. The augmented EPC-mediated neovascularization resulted in a marked improvement of the myocardial microvascularization, which was associated with attenuated MI-induced progression of LV-dilatation and decline in LV function.

Figure 6. Expression of VEGF by bone marrow derived and non-bone marrow derived endothelial cells.



A. Representative fluorescent overlay showing bone marrow derived endothelium (endothelium (his 52, blue) bone marrow derived cells (hPAP, green). **B**. Representative fluorescent overlay showing VEGF expression in the endothelium (endothelium (his 52, blue) VEGF (red)) **C**. Merged picture of A and B showing that VEGF is expressed by bone marrow derived (dashed arrow) and non bone marrow derived (solid arrow) endothelial cells.

In addition, VEGF secretion in the myocardium was increased 4.5-fold by EPO, strongly correlating with neovascularization. VEGF was equally expressed by endothelial cells of myocardial and bone marrow origin, suggesting an additional auto-/paracrine mechanism distinct from bone-marrow-dependent neovascularization.

Erythropoietin prevents left-ventricular-dilatation and left-ventricular dysfunction and regenerates the myocardial microvasculature, associated with endothelial progenitor cell mobilization

In the present study, we confirm that EPO improves cardiac function when administered in the chronic stage after MI. Similar to our previous study,[11] EPO treatment starting 3 weeks after MI had no effect on myocardial infarct size or cardiomyocyte hypertrophy but restored capillary density to sham levels, suggesting that neovascularization is the predominant mechanism through which EPO improves the failing heart. Myocardial remodelling post-MI is associated with impaired perfusion of the noninfarcted LV wall due to disproportionate cardiomyocyte hypertrophy relative to (micro) vascular growth resulting in a progressive deterioration of cardiac function.[24] Our data clearly demonstrate that administration of EPO restores the microvascularization and halts the deterioration of cardiac function. EPO-induced improvement of cardiac function, cardiac neovascularization, and EPC mobilization in post-MI LV-dysfunction was recently confirmed by two independent studies.[9,12] Furthermore, in a distinct model of chronic myocardial dysfunction, EPO prevented doxorubicin induced cardiomyopathy, which was also linked to improved EPC mobilization and capillary density[8,10] Thus, EPO consistently improves cardiac function in experimental models of CHF, strongly linked to increased EPC mobilization and improved microvascularization.

Endothelial progenitor cells mobilized by erythropoietin incorporate into newly formed capillaries

Our data are the first to demonstrate that EPC importantly contribute to EPO-mediated microvascular regeneration of the failing myocardium. The effects of EPO on EPC mobilization were first described by Heeschen *et al.*[18], who also demonstrated an association with neovascularization of ischaemic tissues. Recently, Urao *et al.*[25] were the first to demonstrate that EPO increased incorporation of EPC into the endothelium, in a model of acute aortic-wire injury. However, our data are the first to demonstrate that the EPC mobilized by EPO promote neovascularization of the hypertrophied myocardium in chronic post-MI LV dysfunction. The importance of EPC in EPO-induced cardiac repair has been indirectly signified by three other reports. First, Prunier *et al.* showed that administration of EPO in a dose that does not induce EPC mobilization, failed to improve cardiac function and induce neovascularization, suggesting that EPC-mobilization is required for EPO to improve post-MI myocardial dysfunction.[12] However, EPC were only characterized by expression of CD31, which is also expressed by a variety of leucocytes.[26] Therefore, the data by Prunier *et al.* should be interpreted with caution. Secondly, in a model of doxorubicin induced cardiotoxicity, infusion of isolated EPC prior to doxorubicin infusion, preserved cardiac function in a magnitude equal to EPO.[10] Third, in a model of hypoxia-induced pulmonary hypertension, mice with erythroid restricted expression of the EPO-receptor demonstrated impaired EPC mobilization and augmented disease progression, which was restored by transplantation of wildtype bone marrow.[27] Hence, EPC-mediated neovascularization appears to be crucial for EPO-induced protection from chronic vascular disease.

Erythropoietin increases vascular endothelial growth factor levels and induces expression of its own receptor

Thirty percent of the new endothelium was bone marrow-derived indicating that the remaining new vessels originated from *in situ* proliferated endothelial cells. Both direct effects of EPO on in situ endothelial cell proliferation and paracrine functions of EPC may play a role in this process.[28] In order to investigate whether EPO stimulated the paracrine effect of EPC, we evaluated the expression of the key angiogenic cytokine VEGF. We demonstrate that EPO treatment markedly increases expression of VEGF in the myocardium. Endothelial cells of myocardial and bone marrow origin demonstrated equal VEGF immunoreactivity, which indicates that EPO stimulates secretion of angiogenic factors by endothelial cells in an auto-/paracrine fashion irrespective of their origin. These findings suggest that in addition to EPC-mediated neovascularization, EPO increases *in-situ* proliferation of myocardial endothelial cells, providing a distinct parallel mechanisms of neovascularization. EPO-induced expression of VEGF has also been observed in cultured endothelial cells. Interestingly, neutralization of VEGF stopped the mitogenic effects of EPO in these cultures, suggesting that EPO-induced endothelial cell proliferation is dependent on VEGF.[29,30] Moreover, in addition to auto-/paracrine effects on endothelial cells, myocardial upregulation of VEGF might have chemotactic effects on EPC. The increased VEGF

expression would thereby stimulate homing of EPC into the myocardium, further augmenting EPC-mediated neovascularization. These data suggest an important role for VEGF in EPO-induced neovascularization.

Further studies are needed to evaluate whether VEGF upregulation is a prerequisite for EPO-induced neovascularization. Although the exact role of VEGF remains to be established, our data are the first to demonstrate the broad range of pro-angiogenic effects exerted by EPO, namely EPC-mediated neovascularization, local upregulation of another key angiogenic factor (VEGF), and stimulation of in-situ proliferation of endothelial cells. Furthermore, EPO increased the expression of its own receptor, possibly leading to increased sensitivity to EPO. This has previously been demonstrated in cultured endothelial cells where hypoxia and EPO synergistically induced EPO-receptor expression.[31] These data suggest that EPO regulates EPO-receptor expression through a positive feedback mechanism. Basal expression-levels of EPO-receptor are relatively low in the non-erythropoietic tissues, but a marked up-regulation of the EPO-receptor has been demonstrated by acute metabolic stress, including hypoxia.[32] Therefore, the elevated exogenous EPO levels during treatment might mimic a hypoxia related signal to increase EPO-receptor expression, further potentiating the effects of therapy.

Clinical implications

Our present and previous data clearly show that EPO treatment prevents LV dilatation and myocardial functional decline by regenerating the myocardial microvasculature.

Treatment with EPO has been identified as a promising and safe therapy for acute MI.[33-35] Moreover, correction of anemia in CHF with EPO has proved both safe and feasible.[36] Our findings indicate, however, that EPO exerts beneficial hematocrit independent effects, which might also prove beneficial for non-anaemic CHF patients. Nevertheless, unrestricted dosing regimens of EPO could elevate hematocrit to unacceptable levels, which might lead to impaired rheology, thrombogenicity and hypertension.[37] Indeed, a significant increase in diastolic blood pressure was observed in our study, suggesting EPO-induced hypertension. However, increased arterial pressure might result from the improved myocardial contractility. Systolic and diastolic blood pressure were comparable between the MI–EPO and the sham group, supporting the hypothesis that the increased blood pressure is related to improved function. These limitations might be overcome by using dosing regimens that do not alter hematocrit levels.[38] Alternatively, EPO-derivatives have recently emerged that do not ligate the EPO receptor on erythropoietic cells, but still confer tissue protection.[39] Finally, a very recent study by Schneider et al.[40] revealed that endocardial EPO injections improves the contractile function of hibernating myocardium, without affecting hematocrit levels.

Conclusion

EPO-induced myocardial neovascularization is mediated through a combination of EPC recruitment from the bone marrow and increased VEGF expression in the myocardium.

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Chapter 7

Erythropoietin stimulates normal endothelial progenitor cell-mediated endothelial turnover, but attributes to neovascularization only in the presence of local ischemia

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Abstract

Purpose

We aimed to evaluate whether ischemia is required for erythropoietin (EPO) induced stimulation of endothelial progenitor cells (EPCs) and their related effects on endothelial and cardiac function.

Methods

Bone marrow of rats was replaced by transgenic cells to allow tracking of EPCs. Ischemic heart failure was induced by left coronary artery ligation to induce myocardial infarction (MI) and control rats received a sham procedure. Three weeks after surgery, rats were randomized to receive EPO (darbepoetin alfa 40μg/kg/3weeks) or vehicle and were sacrificed 9 weeks after surgery.

Results

In all treated groups, EPO significantly increased circulating EPCs and their incorporation into the endothelium of the ischemic and non-ischemic hearts as well as in the control organs; kidney and liver. This was associated with significantly improved endothelial function, which was strongly correlated with circulating EPCs ($R=0.7$, $p<0.01$). However, additional EPCs preferentially homed to the ischemic MI borderzone ($p<0.01$) resulting in specific EPO-induced improvement of cardiac microvascularization and performance only in ischemic hearts (all $p<0.05$). The differential stimulation of neovascularization by EPO was associated with increased EPO-receptor and VEGF expression in ischemic hearts only.

Conclusions

In general, EPO stimulates normal endothelial progenitor cell-mediated endothelial turnover, but improves cardiac microvascularization and function only in the presence of ischemia.

Introduction

Erythropoietin (EPO) improves cardiac function and induces neovascularization in experimental models of chronic myocardial dysfunction and is currently evaluated in patients with chronic heart failure.[1-7] EPO-induced neovascularization is related to increased mobilization and incorporation of bone marrow derived endothelial progenitor cells (EPCs) and augmented expression of angiogenic factors in the myocardium.[8] These effects of EPO have also been demonstrated in the endothelium of other ischemic organs, such as the brain and ischemic limbs.[9-12] In addition to new vessel formation, EPCs replace injured endothelium and thereby maintain the integrity of the endothelial monolayer.[13] Since endothelial dysfunction has been recognized as one of the earliest events in atherosclerotic cardiovascular disease[14], stimulation of EPC-mediated endothelial repair by EPO might be of therapeutic interest.

However, the beneficial effects of EPO on endothelial repair and new vessel formation have only been demonstrated in ischemic organs. It is therefore unknown whether these effects are attributable to the presence of ischemia or represent a general phenomenon. We therefore aimed to evaluate whether ischemia is required for erythropoietin (EPO) induced stimulation of endothelial progenitor cells (EPCs) and their related effects on endothelial and cardiac function.

Methods

Animals and bone marrow labeling

For bone marrow transplantation experiments we used male Fischer F344 rats (n=75) weighing 200-230 grams (Charles Rivers, France) as recipients and R26-hPAP donor rats (n=10, F344 background, ubiquitously expressing human placental alkaline phosphatase, hPAP).[15] Details on transplantation have been described in detail previously.[8] Briefly, whole R26-hPAP bone marrow cells were transfused to Fischer F344 recipients after total body irradiation and left to reconstitute for 5 weeks. Leucocyte chimerism was assessed by FACS and rats with chimerism >80% were included in the experimental protocol. Animals were fed and housed, according to institutional rules and regulations. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen. Results of endothelial function were confirmed in Male Sprague Dawley rats weighing 270-320 grams.

Experimental protocol

MI was induced by ligating the left coronary artery.[6] Sham operated rats were used to control for surgery and post-operation inflammation. In sham operated rats, the same surgery was performed without closing the ligature. Three weeks after surgery, rats were randomly assigned to treatment with the long-acting EPO analogue darbepoetin alfa (40 μg/kg, Aranesp, Amgen Inc., Thousand Oaks, CA, USA) or vehicle; once every three weeks, at week 3 and week 6. After 9 weeks, hemodynamic measurements were

performed with a microtip pressure transducer (Millar Instr. Inc., Houston, Texas, USA) as described previously. Heart rate (HR), left ventricular systolic pressure (LVSP), and left ventricular end-diastolic pressure (LVEDP) were measured. The maximal rates of increase and decrease in LVP (dP/dt_{max} and dP/dt_{min}) were determined. Thereafter hearts were rapidly excised and weighed. Myocardial tissue was dissected transversally and processed for immunohistochemistry or snap frozen for Western blot analysis.

Circulating endothelial progenitor cells

Circulating EPCs were enumerated as previously described.[8] Mononuclear cells were isolated from whole blood by density centrifugation and seeded onto fibronectin-precoated 24-well plates (1×10^6 cells per well) in EndoCult medium (StemCell Technologies, London, UK) as described previously. After 6 days of culture, adherent cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labeled acetylated-LDL (DiL-AcLDL) for 12 hours, fixed with 1% paraformaldehyde and counterstained with fluorescein- labeled isolectin B_4. Cells double positive for DiI-AcLDL and lectin staining were considered EPCs and counted in 5 high-power fields, by blinded observers.

Histology

Infarct size and capillary density were determined as described in detail previously.[6] Briefly, infarct size was determined at the mid-papillary level in transverse slices on picrosirius red / fast green–stained sections. Endothelial cells were stained with GSL-Lectin and image analysis was used to measure capillary density and calculated as the number of capillaries per tissue area (mm^2). Cryosections were stained for hPAP (rabbit anti-hPAP, serotec, London, U.K. followed by FITC labeled goat anti-rabbit-IgG) and rat endothelial cell antigen (RECA; mouse anti-rat His52; a kind gift from dr. J.L. Hillebrands, followed by TRITC-labeled goat anti-mouse-IgG-isotype). DAPI was used for nuclear counterstaining. hPAP positive cells were considered bone marrow derived cells (BMDC) and cells double positive for hPAP and His-52 were considered bone marrow derived endothelial like cells (BMDEC). Cells in five random high power fields of the non-infarcted left ventricular free wall were counted independently by two blinded observers.

EPO-receptor and VEGF expression

The expression of EPO-receptor, Vascular Endothelial Growth Factor (VEGF) and GAPDH was determined in tissue homogenates of the viable left ventricular free wall (non infarcted area) by standard Western blotting techniques.[8] Antibodies were purchased from Santa Cruz biotechnology (EPO-receptor M-20, 1:500, VEGF C1, 1:250, (recognizes all splice variants of VEGF), and Fitzgerald Industries (GAPDH 6c5, 1:10000). Horseradish peroxidase conjugated anti-mouse or anti-rabbit IgG were used as secondary antibodies.

Organ bath studies with isolated aortic rings

Vascular measurements were performed as described previously.[16] In brief, thoracic aortic rings were connected to an isotonic displacement transducer and after stabilization rings were checked for viability by stimulation with phenylephrine (1 mmol/L).The endothelium-dependent vasodilatation was assessed by a cumulative dose of metacholine (10^{-9} to 10^{-5} mmol/L). In parallel rings, endothelium-independent vasodilatation was assessed in the presence of NG-Methyl-L-arginine acetate salt (L-NMMA, 10^{-5} mol/L) a nonspecific NOS blocker, or in the presence of indomethacine (10^{-4} mol/L) a nonspecific prostaglandin inhibitor, and finally in the presence of both L-NMMA and indomethacine. Drugs were purchased from Sigma-Aldrich.

Statistical Methods

Data are expressed as means ± SEM. Statistical analysis among groups was performed by ANOVA. Differences in vascular dose-response curves between groups were tested by ANCOVA for repeated measurements. Correlations were assessed with Spearman's correlation test. All P-values were two-tailed, and a P-value of <0.05 was considered statistically significant. All analyses were performed using SPSS version 12.0 software (SPSS, Chicago, IL, USA).

Results

Two sham rats were excluded due to accidental procedural damage to a coronary artery. Mortality after MI was 32%. Hematocrit levels increased from 41 ± 0.5% to 57 ± 0.5 % in EPO treated rats. No differences in hematocrit were observed between sham and MI. At sacrifice, body weight was similar among groups, but heart weight / body weight ratio was significantly increased in the MI groups compared to shams ($p<0.01$). EPO treatment reduced the hypertrophic response after MI (heart weight / body weight ratio: 4.1 ± 1 in MI vs 3.6 ± 2 in MI-EPO $p=0.04$).

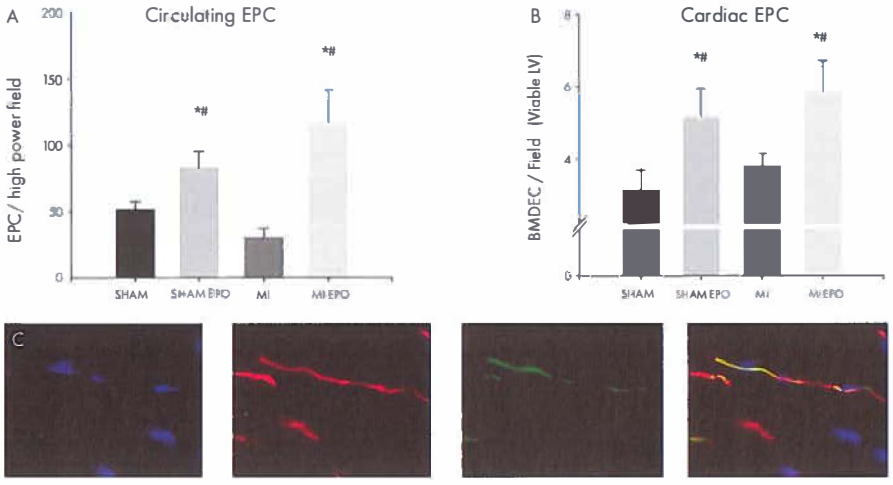
Effects of EPO on circulating EPCs number

In sham rats MI resulted in an almost 60% increase in the number of circulating EPCs (52 ± 6 vs 83 ± 12 per high-powered field in sham vs sham-EPO, $p<0.01$). MI was associated with significantly decreased numbers of circulating EPCs (31 ± 7 in MI, $p<0.01$ vs sham). Treatment with EPO resulted in a substantially more pronounced increase in number of circulating EPCs (31 ± 7 vs 115 ± 22 in MI vs MI-EPO, figure 1A, $p<0.01$). Circulating EPC-numbers did not differ significantly between EPO-treated groups.

Effect of EPO on incorporation of EPCs into the ischemic and non-ischemic myocardium

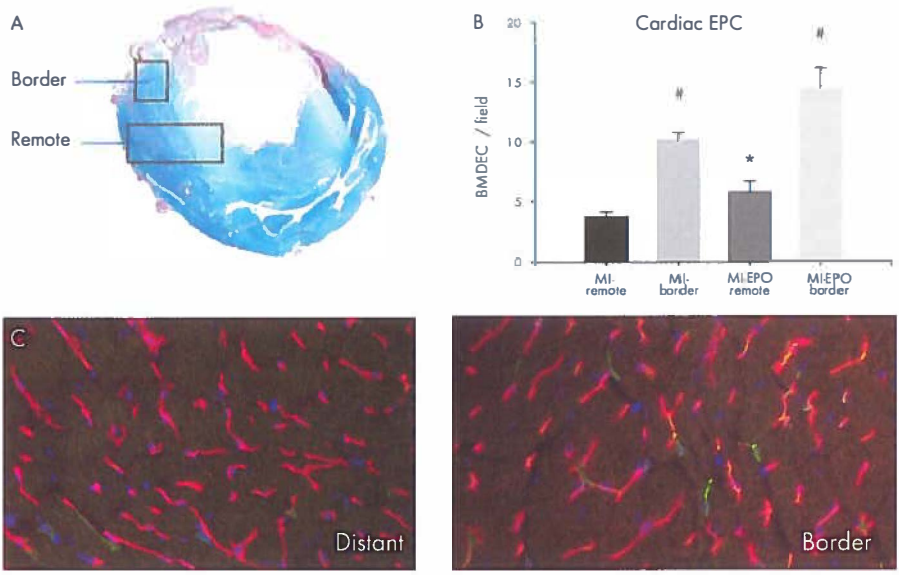
We determined the number of BMDEC in the viable left ventricular free wall, distant from the infracted area, and in the viable borderzone directly adjacent to the infracted area if applicable.

Figure 1. Effect of EPO on circulating and cardiac Endothelial Progenitor Cells.



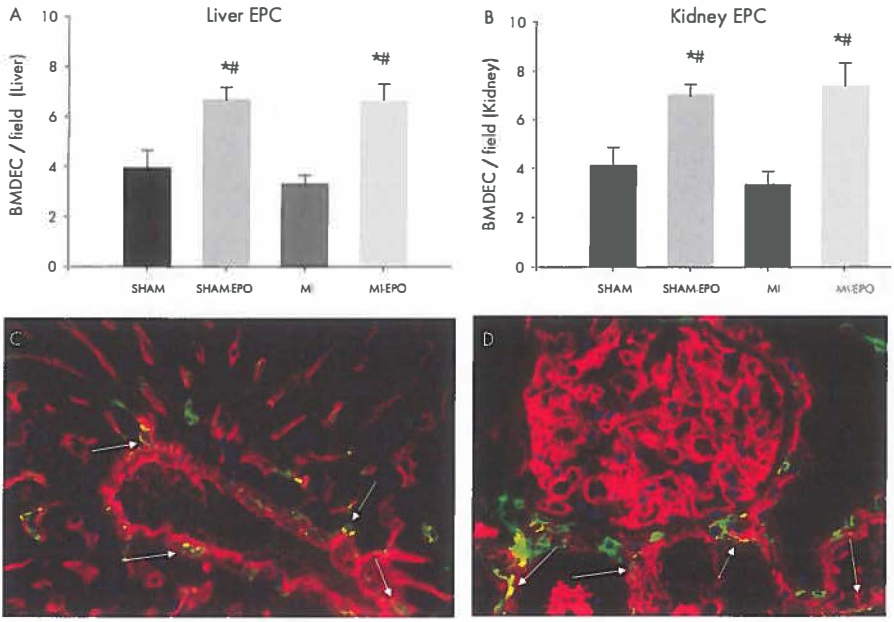
A. Graphic representation of the number of circulating EPCs. **B**. Bar graph representing the number of Bone Marrow derived Endothelial Cells (BMDEC, cells staining double positive for hPAP and His 52) in the viable left ventricular free wall distant from the infarct (if applicable). **C**. Typical BMDEC , myocardial section stained with hPAP (green), His 52 (endothelium, red) and DAPI (nucleus, blue). The three panels display respectively nuclei, endothelium, bone marrow derived cells and the overlay of the 3 channels, where BMDEC appear yellow. * p<0.05 vs sham, # p<0.05 vs MI.

Figure 2. Effect of ischemia on homing of Endothelial Progenitor Cells.



A. Typical myocardial section showing infarct scar (red), viable myocardium (blue) and panels showing the areas considered infarct borderzone (border) and viable left ventricular (LV) free wall remote from the infarct (remote). **B**. Bar graph representing the difference between BMEDC in the barderzone and the remote LV-wall. **C**. Representative fluorescent overlay of the borderzane and the remote LV. * p<0.05 vs sham, # p<0.05 vs MI.

Figure 3. Effect of EPO on incorporation of Endothelial Progenitor Cells in the liver and the kidney.



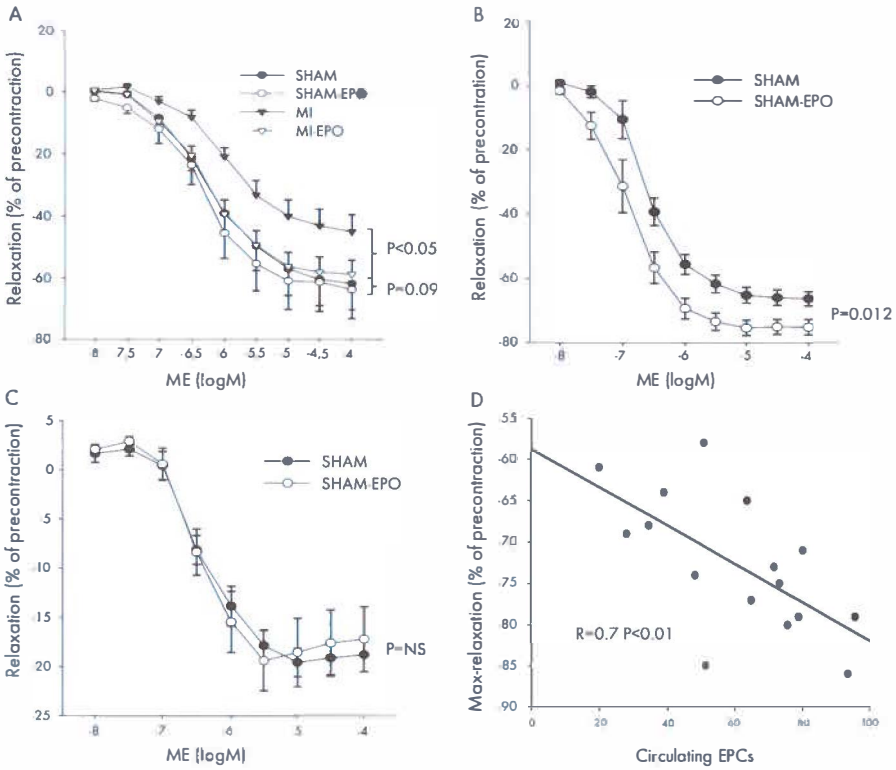
A. Bar graph representing the number of bone marrow derived endothelial cells (BMDEC) in the liver. **B.** Bar graph representing the number of BMDEC in the kidney. **C.** Representative fluorescent overlay of a liver section BMDEC incorporated into the endothelium which appear yellow (40x magnification). BMDEC are indicated with a white arrow. **D.** Representative fluorescent overlay of a kidney section showing bone marrow derived cells incorporated in the interstitium (green) and the endothelium (yellow) surrounding the glomerulus (40x magnification) BMDEC are indicated with a white arrow.* $p<0.05$ vs sham, # $p<0.05$ vs MI.

In the left ventricular free wall, the number of BMDEC was comparable between sham and MI rats (3.2 ± 0.6 vs 3.8 ± 0.3 BMDEC per high-powered field, $p = 0.5$, figure 1B). EPO treatment almost doubled the number of BMDEC after MI in the left ventricular free wall, but to a similar extent after sham operation (5.8 ± 0.9 in MI-EPO and 5.0 ± 0.9 in sham-EPO both $p<0.05$, figure 1B). The number of BMDEC in the borderzone was significantly higher compared to the left ventricular free wall in both MI groups (3.8 ± 0.3 vs 10 ± 0.6 in MI and 5.8 ± 0.9 vs 15 ± 1.7 in MI EPO, both $p<0.01$ figure 2), indicating preferential homing of EPCs into the ischemic myocardium. Ischemia-induced homing of EPCs to the MI-borderzone was also markedly augmented by EPO-induced EPC mobilization ($p<0.05$).

Effect of EPO on incorporation of EPCs in endothelium of other organs

The number of BMDEC in kidneys and livers were comparable in sham and MI rats (figure 3). EPO treatment resulted in a significantly increased incorporation of BMDC in the endothelium of kidneys and livers in both sham and MI rats (figure 3).

Figure 4. Effects of EPO on endothelial function.



A. Endothelium dependent vasodilatation to methacholine (ME) of each group of bone marrow transplanted Fischer F344 rats, expressed as the percentage of displacement induced by phenylephrine precontraction. **B**. Endothelium dependent vasodilatation to ME in Sprague Dawely sham operated rats. **C**. Endothelium dependent vasodilatation to ME in Sprague Dawely rats with concomitant NO-blockade with L-NMMA. **D**. Correlation between maximal relaxation to ME and the number of circulating EPC in Sprague Dawely rats.

To assess the relationship between circulating EPCs and incorporation of BMDEC, we analyzed all groups combined. A significant correlation between circulating EPC-number and incorporated number of BMDEC were observed in the heart ($R=0.5$, $p<0.01$), kidney ($R=0.6$, $p<0.01$), and liver ($R=0.56$, $p=0.02$).

Effect of EPO on endothelial function

Endothelial-dependent vasodilatation in response to metacholine is depicted in figure 4. Induction of MI resulted in a significant impairment of endothelial-dependent vasodilatation ($p<0.01$, figure 4A). EPO treatment significantly improved endothelial dependent relaxation in MI rats, almost restoring it to sham levels ($p<0.05$ vs MI). Interestingly, EPO also tended to improve endothelial dependent vasodilatation in sham rats ($p=0.09$ vs sham). To assess the mechanisms involved and to exclude

potential effects of irradiation on endothelial function parameters, we also assessed vascular function in male Sprague Dawley rats. In these experiments we confirmed that EPO treatment resulted in an improvement of endothelial dependent vasodilatation ($p=0.012$, figure 4B). The effect of EPO on endothelial dependent-vasodilatation was completely abrogated by co-incubation with L-NMMA, suggesting involvement of eNOS (figure 4C). Blockade of prostacyclins by indomethacin did not significantly change vasodilatation (data not shown). To assess the relationship between circulating EPCs and endothelial function we correlated the number of circulating EPCs and the maximal rate of metacholine induced relaxation. A strong correlation was observed ($R=0.7$, $p<0.01$, Figure 4D).

Effect of EPO on myocardial capillary density

Induction of MI significantly reduced cardiac capillary density ($p=0.01$ versus sham, figure 5A). EPO significantly increased capillary density in MI rats, almost restoring it to sham levels($p<0.01$ vs. MI; and p=NS vs sham, figure 5A). In parallel, EPO treatment resulted in a significant increase in the protein expression of EPO-receptor and VEGF in MI rats ($p<0.01$, figure 5C and 5D). However, EPO treatment did not affect myocardial capillary density, EPO-receptor or VEGF expression in sham rats.

Effect of EPO on cardiac function

Cardiac function was significantly reduced in MI groups compared to sham (all $p<0.05$, figure 6). EPO treatment significantly increased myocardial contractility, relaxation and LVESP and a decrease in LVEDP in MI rats, but not in shams (figure 6)

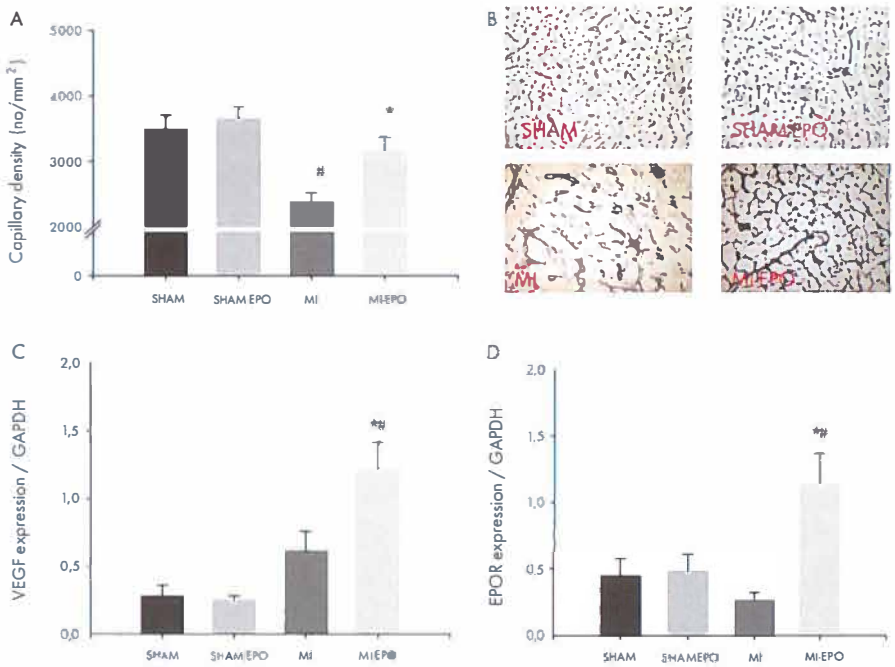
Discussion

In the present study we demonstrate for the first time that the regenerative effects of EPO in chronic heart failure are dependent on the presence of ischemia. EPO stimulated mobilization of EPCs from the bone marrow which indiscriminately incorporated in the endothelium of ischemic and non-ischemic tissues and significantly improved endothelial function. However, EPO stimulated neovascularization and improved cardiac function only in ischemic hearts, which was possibly driven by augmented local VEGF and EPO-receptor expression. These results suggest that EPO regulates normal endothelial progenitor cell-mediated endothelial turnover, but improves microvascularization and cardiac function only in the presence of ischemia. The differential effects of EPO in the presence and absence of ischemia are summarized in table 1.

EPO augments EPC-mediated endothelial turnover

The endothelium is recognized to play a crucial role in atherogenesis and considered to be involved early in the pathophysiology of cardiovascular disease.[14] In patients with essential hypertension and documented coronary artery disease, endothelial

Figure 5. Effects of EPO on neovascularization and expression of pro-angiogenic factors in ischemic and non-ischemic hearts.

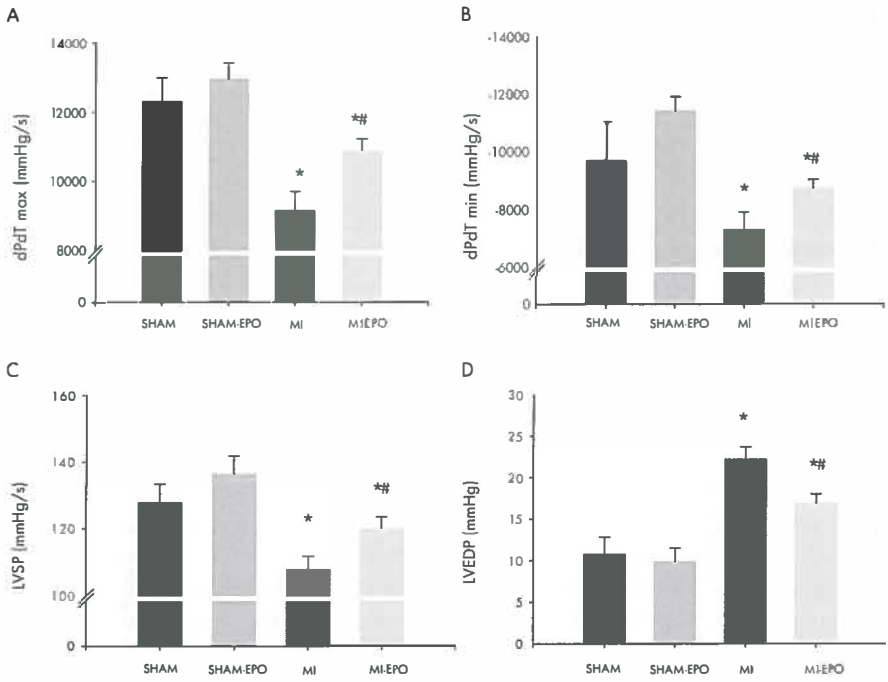


A. Measurements of capillary density expresses as the number of capillaries /mm². **B**. typical examples of capillary density in the experimental groups. **C**. Protein expression of VEGF corrected for GAPDH in the left ventricular free wall. **D**. Protein expression of EPO-receptor corrected for GAPDH in the left ventricular free wall. * p<0.05 vs sham, # p<0.05 vs MI.

dysfunction appears a marker for future cardiovascular events.[17] Moreover, chronic heart failure is associated with endothelial dysfunction which promotes cardiac remodeling through increased peripheral resistance, eventually reducing prognosis.[18] Maintaining a vital endothelial monolayer is therefore of pivotal importance in the prevention and treatment of cardiovascular disease. Endothelial turnover can be facilitated through migration and proliferation of neighboring endothelial cells, although the rate of endothelial-cell proliferation in arteries is relatively low.[19] In contrast, EPCs rapidly repopulate denuded arteries, associated with an immediate improvement of endothelial function.[20] Thus in addition to their role in neovascularization, EPCs serve as gatekeepers for endothelial patency and function.

Urao et al. were the first to demonstrate that EPO does not only induce EPC mobilization but also promotes incorporation of EPCs into sites of endothelial injury.[12] In addition, we recently revealed that EPO-induced neovascularization in chronic post-MI heart failure is at least partially mediated through increased incorporation of EPCs into the myocardial microvasculature.[8] The present study is however the first to

Figure 6. Effects of EPO on indices of left ventricular function.



A. Graphic representation of left ventricular contractility (dPdT max), **B**. Graphic representation of left ventricular relaxation(dPdT min). **C**. Graphic representation of left ventricular systolic pressure (LVSP). **D**.Grophic representation of left ventricular systolic end diastolic pressure (LVEDP). * $p<0.05$ vs sham, # $p<0.05$ vs MI.

demonstrate that increased EPC-mobilization by EPO augments incorporation of EPCs into the endothelium, independent of the presence of ischemia. Therefore, our results substantiate the hypothesis that augmented EPC-mobilization by EPO accelerates endothelial turnover.

The increased endothelial turnover was associated with restoration of heart failure-induced deterioration of endothelial function. Moreover, although we consider endothelial function relatively normal in sham-operated rats, EPO treatment further improved endothelial dependent relaxation, through augmented release of NO. Interestingly, in normal rats a strong correlation was observed between maximal rates of endothelial dependent relaxation and the number of circulating EPC, substantiating a direct relationship between EPC-mediated endothelial turnover and vascular function outside the setting of an vascular injury. It is tempting to speculate that the improvement of endothelial function is caused by the replenishment of functionally impaired endothelial cells by EPCs. Alternatively, paracrine effects of incorporated EPCs might contribute to functional improvement of surrounding cells.[20]

Table 1. Effects of erythropoietin in the presence or absence of ischemia.

Effects of erythropoietin	Absence of ischemia	Presence of ischemia
EPCs mobilization	+	+
Vascular incorporation of EPCs	+	++
Endothelial function	+	+
Neovascularization	-	+
Cardiac function	-	+
EPO-receptor expression	-	+
VEGF expression	-	+

EPO, erythropoietin; VEGF, Vascular Endothelial Growth Factor; +, increased by EPO, -, not increased by EPO, ++, markedly increased by EPO.

Direct effects of EPO on resident endothelial cells should also be considered, since EPO has been shown to activate endothelial cells in vitro and in vivo, associated with eNOS up regulation and improved endothelial function.[21, 22] However, resident endothelial cells are relatively insensitive to EPO while EPCs respond to EPO-doses that are insufficient to activate erythropoietic cells.[9, 23] Future studies are required to differentiate between direct and indirect effects of EPO on endothelial function.

EPCs switch from endothelial turnover to EPO-induced neovascularization in the presence of ischemia

After MI, perfusion of the remaining vital myocardium will become insufficient due to disproportionate cardiomyocyte hypertrophy relative to (micro) vascular growth, resulting in ischemia and up regulation of hypoxia inducible factors.[74, 75] Although non-specific incorporation of EPCs into the systemic endothelium was observed, EPCs preferentially homed to ischemic hearts and even more specifically to the regions with the most profound perfusion insufficiency. The specific homing of EPCs into ischemic myocardium was associated with differential induction of neovascularization, only in rats with ischemia due to post-MI heart failure. Hence, the presence of ischemia seems necessary to switch from vascular "maintenance" to EPO-induced neovascularization.

VEGF levels were slightly increased in the untreated MI group, but EPO treatment augmented VEGF expression 4-fold. This effect of EPO could not be extended to sham operated rats without cardiac ischemia. Upregulation of VEGF at sites of EPO-induced neovascularization in ischemic tissues has been substantiated in other reports.[11,26] Moreover, in mice that lack EPO-receptor expression in the vasculature, VEGF upregulation in the ischemic hind limb is markedly attenuated and associated with significantly reduced neovascularization and homing of EPCs.[27] VEGF is a potent angiogenic factor that stimulates in situ proliferation of endothelial cells and has important chemotactic effects on EPCs.[28] Therefore, EPO-induced expression of

VEGF might mediate the preferential homing of EPCs into the ischemic tissue, thus facilitating EPC-mediated neovascularization. However, the increased VEGF expression might also stimulate resident endothelial cells and thereby induce neovascularization through EPC-independent mechanisms.

EPO-induced VEGF upregulation is mediated through EPOR signaling pathways.[26,27,29] In addition to VEGF upregulation, EPO was associated with augmented EPOR expression in the MI but not in the sham group, which confirms similar findings by others.[30] Extra-hematopoietic tissues are relatively insensitive to EPO, and binding affinities for EPO are 10-fold lower in endothelial cells compared to erythropoietic progenitors.[23] The combined presence of hypoxia and ischemia however significantly augments sensitivity to EPO through an exponential increase in EPO-receptor expression.[30] Separately these factors exert minute effects. Therefore, the absence of EPO-induced VEGF expression and neovascularization might in part be explained through absence of ischemia-induced sensitization to EPO.

Clinical implications

In the present study, we demonstrate that EPO treatment might reverse endothelial dysfunction in the early stages of cardiovascular disease and thereby prevent the progression into cardiovascular events. This important role for EPO in the regulation of endothelial cell turnover and endothelial function, might in part explain the high incidence of vascular events in patients with EPO deficiency due to chronic kidney disease. The beneficial effects of EPO treatment in these patients may not only result from the correction of anemia but from an improved endothelial function as well. Moreover, restoration of endothelial dysfunction in patients with heart failure will decrease peripheral resistance and thereby reduce cardiac afterload and possibly improve outcome as well. The improvement of endothelial function in our study was mediated through augmented endothelial NO-production. In addition to vasodilatation, augmented NO-production has been identified as an important compensatory mechanism against the pro-trombotic effects of EPO.[21] A possible therapeutic role for EPO in its present form in non-anemic patients is however complicated by the unrestricted elevation of hematocrit. The safety of which is currently also questioned in patients with chronic kidney disease and cancer.[31, 32] Recently we have demonstrated that cardiac function in post-MI heart failure can be augmented with an EPO dose that does not increase hematocrit levels. This might suggest that even non-erythropoeitic doses of EPO will benefit patients.[33] Ideally, a minimally effective dose should be established which exerts optimal benefit with little or no effects on hematocrit. In addition, non-erythropoietic EPO derivatives have emerged.[34] These strategies might provide us with tools to revitalize the endothelium of patients at risk of major cardiovascular events. Care should however be taken not to over stimulate the bone marrow as this may lead to functional exhaustion the bone-marrow stem cell population.[35, 36]

Conclusions

In general, EPO stimulates normal endothelial progenitor cell-mediated endothelial turnover. However, EPO attributes to neovascularization and improves cardiac function only in the presence of local ischemia.

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Chapter 8

Low-dose erythropoietin improves cardiac function in experimental heart failure without increasing hematocrit

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Abstract

Background

Erythropoietin (EPO) may improve cardiac function and induce neovascularization in experimental models of chronic heart failure (CHF). However, the increased hematocrit associated with EPO treatment might exert concomitant deleterious effects.
We aimed to investigate the hematocrit independent effects of EPO on cardiac function.

Methods and results

Rats were subjected to permanent coronary artery ligation to induce myocardial infarction (MI) or sham surgery. Three weeks after MI, rats were randomly allocated to treatment with vehicle (MI) or the long-acting EPO analogue darbepoetin alfa in a high (40 μg/kg/3 weeks, MI-EPO-high) or a low dose (0.4 μg/kg/3 weeks, MI-EPO-low). After 9 weeks, hemodynamic parameters, myocardial histology and Myocin Heavy Chain (MHC) isoforms were determined. High dose EPO resulted in a significant increase in hematocrit ($p<0.01$) while low dose EPO had no effect on hematocrit levels. EPO significantly improved cardiac function in both EPO groups, reflected by increased LV-developed pressure and improved contractility (dP/dt_{max}) and relaxation (dP/dt_{min}) indices of the LV at 9-weeks (all $p<0.05$ compared to MI). The improved cardiac function was associated with an increased capillary growth (38% in MI-EPO-high ($p<0.01$) and 27% in MI-EPO-low ($p<0.05$)) and an attenuated switch to slow β-MHC isoforms in both EPO-treated groups.

Conclusions

EPO improves cardiac function and induces neovascularization in a dose that does not increase hematocrit, thereby circumventing the possible deleterious effects of increased erythropoiesis.

Introduction

The classical role of erythropoietin (EPO) is related to its hematopoietic effects. EPO is produced in kidneys and acts as a major regulator of erythropoiesis, by increasing survival and promoting proliferation of erythroid progenitor cells.

However, EPO has recently been shown to render organ protection in various experimental models of acute ischemia, including stroke and myocardial infarction, mainly through a reduction of apoptotic cell death.[1-4] In addition, EPO improves cardiac function in experimental models of chronic myocardial dysfunction, which is consistently associated with improved microvascularization of the myocardium.[5-10] The neovascularization by EPO is associated with marked mobilisation and vascular incorporation of endothelial progenitor cells (EPC).[11-14] In addition, EPO stimulates neovascularization by inducing direct mitogenic effects on endothelial cells through local upregulation of VEGF.[14-16] Therefore, the ancillary properties of EPO seem independent of erythropoiesis.

However, the effects of EPO in chronic myocardial dysfunction have been established with repetitive dosing regimens that significantly increased hematocrit levels. Therefore, they might at least to some extend, be related to the increased oxygen-carrying capacity of blood. Moreover, EPO treatment in non-anemic patients could lead to unwanted elevation of hematocrit, associated with higher risk for thrombosis and hypertension.[17] Indeed, the recently published CREATE and the CHOIR study revealed that overcorrection of anemia with recombinant human EPO in patients with chronic kidney disease (CKD) was associated with increased cardiovascular events.[18,19] In Chronic Heart Failure (CHF) patients, appropriately powered phase-3 studies are lacking, although safety and feasibility studies demonstrated promising beneficial effects without additional harm.[20] Nevertheless, treatment aimed at improving cardiac function in non-anemic CHF patients at already elevated cardiovascular risk necessitates treatment strategies that exert the beneficial effects of EPO without increasing hematocrit levels.

The erythropoietin receptor in the heart is structurally and functionally distinct from it's hematopoietic counterpart, and can be specifically targeted.[21] Moreover, chronic EPO administration in a dose that was insufficient to increase hematocrit, improved the survival, ameliorated endothelial damage and preserved renal function in a rat remnant kidney model, suggesting a different dose-response relationship for the erythropoietic and pleiotropic effects.[22] Low-dose EPO might therefore exert similar beneficial effects on the myocardium, without the deleterious effects on hematocrit.

We hypothesized that the ancillary properties of EPO in CHF are independent of the effects on erythropoiesis, and can be induced with an EPO dose that does not increase hematocrit. We therefore studied the effects of high- and low-dose EPO treatment on cardiac function, neovascularization and myosin heavy chain (MHC) isoform expression in an experimental model of post-MI heart failure.

Methods

Animals

Male Sprague Dawley rats weighing 270-330 g (Harlan, Zeist, The Netherlands). Animals were fed ad libitum, and housed in groups of four to five rats, according to institutional rules with 12:12 hours light-dark cycles. The experimental protocol was approved by the Animal Ethical Committee of the University Medical Center Groningen.

Design of the study

Rats were either subjected to left coronary artery ligation (n=63) or sham surgery (n=11). Rats with MI were randomly allocated to 3 groups: control (MI) and two EPO treatment groups with different dosages of long-acting EPO analogue darbepoetin: 40 μg/kg (MI-EPO-high) and 0.4 μg/kg (MI-EPO-low). Darbepoetin-alpha (Aranesp, Amgen Inc., Thousands Oaks, CA, USA) was administered intraperitoneally, once every three weeks, starting three weeks after the coronary artery ligation, hence after the healing phase of MI. Control (MI) and SHAM rats received corresponding injections of saline. The high dose of darbepoetin was based on our previous study [10], demonstrating increased neovascularization in this model, together with significant elevation of hematocrit levels. To avoid the effect of EPO treatment on hematocrit we included a low-dose EPO group, with 100-times lower darbepoetin dosage (0.4 μg/kg/3 weeks), which in a pilot experiment did not cause elevation of hematocrit (data not shown). Hematocrit was measured at baseline and at week 3, 4, 6 and 9 after surgery.

Myocardial infarction model

This model has been described previously.[23] Briefly, rats were anesthetized with 2.5% isoflurane and placed on a heating pad (37°C). Animals were intubated and mechanically ventilated using room air enriched with 1.0 l/min oxygen. After left-side thoracotomy, MI was induced by ligating the proximal portion of the left coronary artery, beneath the left atrial appendage. In sham operated rats, the same surgery was performed, without ligating the suture.

Hemodynamic measurements

After nine weeks, rats were anesthetized as described above. Microtip pressure transducer (Millar Instr. Inc., Houston, Texas, USA) was inserted into the left ventricular cavity via the right carotid artery. After a 3-min period of stabilization, heart rate (HR), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), and developed left ventricular pressure (dLVP=LVSP-LVEDP) were measured. As indices of contractility and relaxation, the maximal rates of increase and decrease in LVP (dP/dt_{max} and dP/dt_{min}) were determined. The catheter was retracted into the aortic arch and arterial systolic and diastolic blood pressures (SBP, DBP) were recorded.

Infarct size and myocyte hypertrophy

After hemodynamic measurements, hearts were rapidly excised and weighed. Mid-papillary slices were fixed in 4% paraformaldehyde and paraffin-embedded. Infarct size was determined by planimeter in transverse slices on picrosirius red/fast green–stained sections. Infarct size was expressed as the percentage of scar length to total left ventricular circumference, as previously described.[24] Deparaffinised 5-μm thick sections were stained with a Gomori's silver staining. Using image analysis (Zeiss KS 400, Germany), concentric myocyte hypertrophy in the viable LV wall, remote from the infarcted area, was measured as the cross-sectional area of transversally cut myocytes showing a nucleus.[23] Myocyte density was calculated as the average number of myocytes per tissue area (mm^2). In each stained section, measurements were averaged from three different counting fields (± 75 myocytes per heart)

Capillary density

To visualize the capillaries in the myocardium of the LV free wall, endothelial cells were stained with biotin-labeled Lectin GSL (1:100; Sigma-Aldrich, St. Louis, Missouri, USA), as previously described. Since lectins stain not only capillaries but other vessels as well, a size criterion of 10 μm was used to exclude small arterioles and venules. Image analysis (Image-Pro Plus for Windows, version 4.5.0.29) was used to measure capillary density, calculated as the number of capillaries per tissue area (mm^2). As a measure of neovascularization, capillary-to-myocyte ratio was calculated dividing capillary with myocyte density, as previously described.[23]

Myosin heavy chain (MHC) isoform analysis

As a molecular marker for changes in myocardial contractility, myosin heavy chain (MHC) isoform analysis was performed. Samples of the non-infarcted left ventricular free wall were snap frozen and stored at -80°C until analysis. Gel electrophoresis was performed on tissue lysates as described previously [25]. In brief, samples were run at constant current (24 mA) for 5 h. Hereafter silver staining was performed and the percentage fast α-MHC vs. slow β-MHC was determined with laser scanning densitometry. Increased expression of slow β-MHC results in impaired contractility of cardiomyocytes and represents a molecular marker of impaired contractility.

Apoptosis analysis

To visualize apoptotic cells in the myocardium, deparaffinised sections were stained with a monoclonal anti-cleaved caspase-3 antibody (ASP175, cell signaling technologies, MA, USA) and visualized with the ENVISION kit (DAKO Cytomation, Glostrup, Denmark) according to the guidelines provided by the supplier. Cleaved caspase-3 positive cells were considered apoptotic and were expressed per 10.000 cells.

Statistical analysis

Data are presented as mean ± SEM, or as median ± IQR (25^{th} and 75^{th} percentile) depending on their distribution. Differences among groups were tested using one-

way analysis of variance, followed by LSD post-hoc analysis if normally distributed, and by Kruskal-Wallis test if skewed distributed. Correlation analysis was performed with Spearman's correlation test. All reported probability values were 2-tailed, and a p-value <0.05 was considered statistically significant. All statistical analyses were performed with SPSS version 11.0.

Results

General characteristics

Overall 24-hour mortality following MI was 41%. Five additional MI rats died during follow-up (2 in MI group, 1 in MI-EPO-high and 2 in MI-EPO-low group).

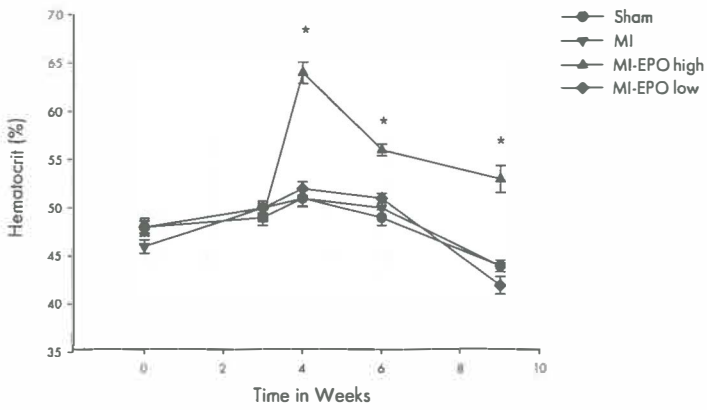
Two rats (1 in MI and 1 in MI-EPO-high group) had infarct size < 25%. These were excluded from further analysis. General characteristics after nine weeks are shown in table 1. LV-infarct size (% of LV) was comparable between all MI groups (table 1). Body weight (BW) was significantly higher only in the MI-EPO-low group (table 1). The heart weight to BW ratio was significantly increased in the rats with MI compared to the sham rats (all $p<0.05$; table 1). A lower heart weight to BW compared to MI group was observed in MI-EPO-high and MI-EPO-low groups (both $p<0.05$).

Table 1. Characteristics of the experimental groups at sacrifice (9 weeks).

	Sham	MI	MI-EPO-High	MI-EPO-Low
General				
n	11	10	11	9
Infarct size	-	45±3	47±2	50±3
Hemodynamics				
Heart rate (bpm)	321±6	318±8	339±7	312±7
LVSP (mmHg)	130±3	104±6†	119±3*§	115±4†
LVEDP (mmHg)	10±1	24±4†	16±2‡	19±2*
SBP (mmHg)	127±3	102±5†	115±3*‡	113±4*
DBP (mmHg)	81±2	73±3*	83±3§	80±2
Body/organ weight				
BW (g)	410±6	402±12	416±8	442±8*§
Heart weight/BW(mg/g)	4.5±0.1	6.0±0.3†	5.3±0.1†‡	5.3±0.3*‡

Data are presented as mean ± SEM; n indicates number of animals; bpm, beats per minute; LVSP, left ventricular systolic pressure; LVEDP, left ventricular end-diastolic pressure; SBP, systolic blood pressure; DBP, diastolic blood pressure; BW, bodyweight. *$p<0.05$; †$p<0.01$ vs. Sham; ‡$p<0.05$, §$p<0.01$ vs. MI.

Figure 1. Effect of EPO treatment on longitudinal changes in hematocrit.



*p<0.01 vs. sham.

Effects of EPO on hematocrit

The changes of the hematocrit throughout the experiment are shown in figure 1. Only the treatment with high-dose EPO led to significant increase in hematocrit levels, which persisted throughout the experiment. Importantly, hematocrit levels in MI-EPO-low group were similar to those of MI and sham groups.

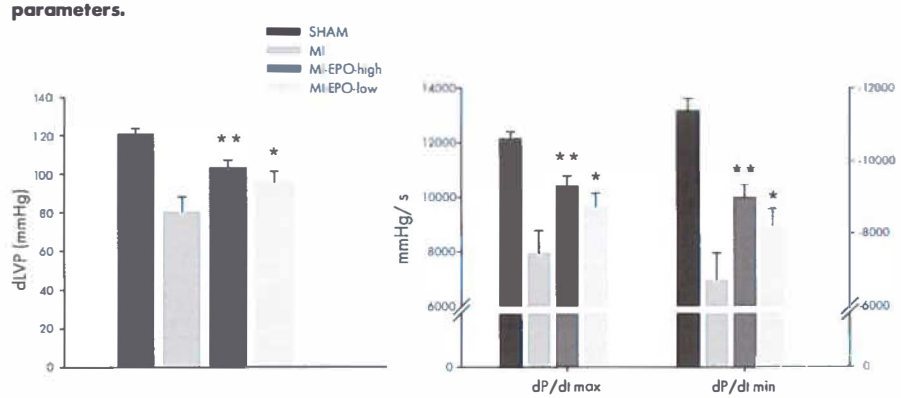
Effects of EPO on cardiac function

Invasive pressure measurements were performed 9 weeks after the surgery immediately before the rats were sacrificed. Myocardial contractility (dP/dt_{max}) and myocardial relaxation (dP/dt_{min}) were both impaired in all MI groups compared to the sham group (all $p<0.05$). Both low- and high-dose EPO treatments resulted in improved contractility and relaxation compared to MI (bothl $p<0.05$; figure 2). LVSP and developed LVP (dLVP) were both decreased in all MI groups compared to sham operated rats ($p<0.05$ for all). MI-EPO-high showed a significantly higher LVSP and dLVP (table 1), compared to MI (both $p<0.01$). Low-dose EPO resulted in a 17% higher dLVP ($p<0.05$), and a trend towards elevation of LVSP, compared to the control group ($p=0.07$; table 1). LVEDP was elevated in the MI-group compared to the sham operated rats ($p<0.01$; table 1). Compared to the MI group, LVEDP was 34% ($p<0.05$) lower In the MI-EPO-high group and 20% lower in in the MI-EPO-low group (p=NS). SBP and DBP were higher only in the MI-EPO-high group, compared to control (both $p<0.05$).

Effects of EPO on neovascularization

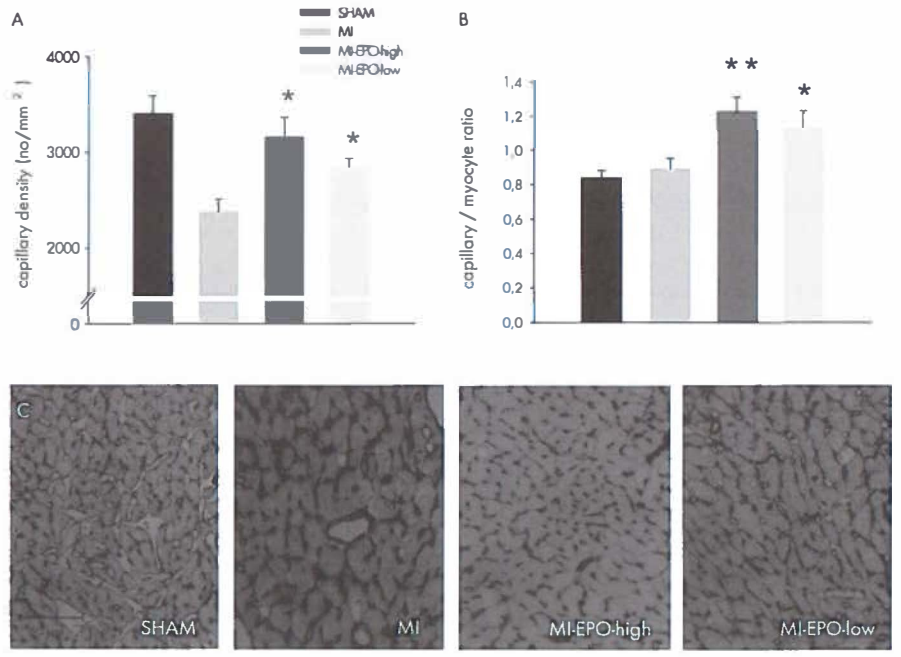
Figure 3C shows representative photomicrographs of the four different groups. Capillary density was significantly reduced in MI compared to sham-group ($p<0.01$). High-dose EPO treatment prevented the decrease in capillary density after induction of MI and restored it to sham values, as shown in Figure 3A (p=NS vs. sham).

Figure 2. Effects of myocardial infarction and different doses of EPO treatment on hemodynamic parameters.



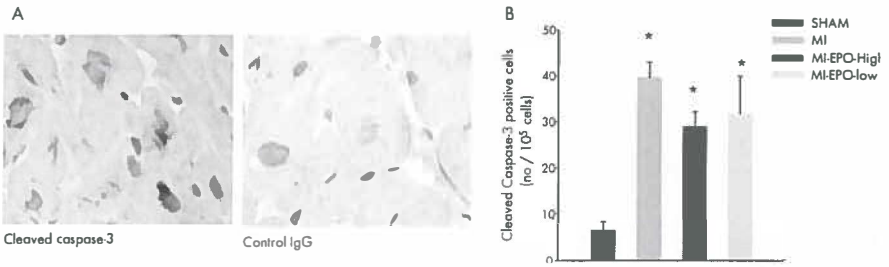
dP/dt_{max} and dP/dt_{min}, maximal rate of increase and decrease of ventricular pressure, respectively. * p<0.05 vs. MI, ** p<0.01 vs. MI.

Figure 3. Effect of EPO treatment on neovascularization.



A. Actual measurements of capillary density in number of capillaries per mm². **B**. Bar graph representing the capillary-to-myocyte ratio in different groups. **C**. Tissue sections with lectin in the viable free wall of the four different groups, showing individual capillaries. *p<0.05 vs. MI, ** p<0.01 vs. MI.

Figure 4. Effects of EPO on myocardial apoptosis.



A. Immunohistochemical staining of a cleaved caspase-3 positive cardiomyocyte under high power magnification showing a nucleus (blue) and cytoplasmatic presence of cleaved caspase 3 (brown, upper panel). Rabbit immunoglobulin IgG was used as a negative control (lower pannel). **B**. Graphic representation of the number of cleaved caspase-3 positive cells in the viable myocardium. *p<0.01 vs. sham.

In this group (MI-EPO-high) we observed a 33% increase in capillary density compared to MI group ($p<0.01$). Treatment with low-dose EPO resulted in a 20% higher capillary density ($p<0.05$). The cross-sectional area of cardiomyocytes increased in all MI groups compared to sham, although EPO treatment had no effect on cardiomyocyte hyperthrophy. Compared to MI group, the capillary-to-myocyte ratio increased by 39% in MI-EPO-high ($p<0.01$) and by 27% in MI-EPO-low ($p<0.05$) (figure 3B). The differences between MI-EPO-high and MI-EPO-low group were not statistically significant.

Effects of EPO on MHC isoforms

Induction of heart failure resulted in a 2.5 fold increase in the expression of β-MHC in the MI group (24 c 5 % vs. 58 ± 5 % in sham vs. MI, figure 4). EPO significantly attenuated the shift from α-MHC to β-MHC (figure 5, $p<0.01$) by 31 % in MI-EPO high (40 ± 3 %) and by 28 % in the MI-EPO low group (42 ± 4 %).

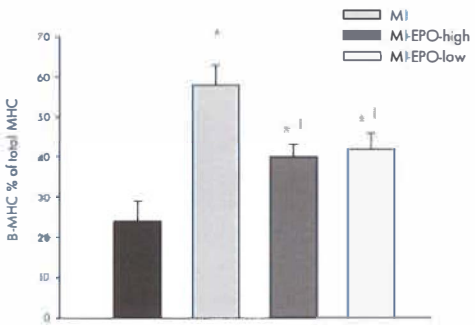
Effects of EPO on myocardial apoptosis

The number of cleaved caspase-3 positive cells was significantly higher rats with heart failure, compared to shams (6.6 ± 2, 40 ± 3.4 , 29 ± 3, 32 ± 8 in sham, MI, MI-EPO-high and MI-EPO-low respectively, $p<0.01$, figure 4). However, the numbers of cleaved caspase-3 positive cells did not differ significantly between the MI groups.

Discussion

In the present study we demonstrated that the beneficial effects of EPO on cardiac function in post-MI heart failure are at least in part independent of an increased hematocrit. EPO treatment in a dose that was insufficient to raise hematocrit levels,

Figure 5. Effect of EPO treatment on β-myocin heavy chain (MHC) expression.



Graphic representation of the expression of β-MHC as a percentage of total MHC. *p<0.001 vs. sham ‡ p<0.01 vs. MI.

markedly improved cardiac function.The functional improvement was associated with induction of neovascularization, and attenuated expression of slow β-MHC isoforms. Most of the beneficial effects were slightly less pronounced in the low dose group, which might indicate that part of the beneficial effects are related to an increased hematocrit. Alternatively, it might reflect the dose dependent nature of the pleiotropic effects of EPO on the heart, which are completely distinct from erythropoiesis.

In the clinical setting, current therapy after MI is focused on prevention of ventricular remodelling and development of heart failure. Myocardial regeneration may offer possibilities that could improve cardiac function in these patients.[26] Although cardiomyocytes proliferation after ischemic injury seems limited, the formation of new vessels in the non-infarcted part of the ventricle could lead to an improvement of function and attenuation of ventricular remodelling.[27,28] Evidence is accumulating to suggest that EPO exerts potent pleiotropic effects on the myocardium in the setting of acute myocardial infarction and chronic heart failure as well.[2,5-10,29] Therefore, in addition to it's acute protective effects, EPO might evolve as a standard "cardioregenerative" therapy in the setting of chronic myocardial dysfunction.

The dosing regimens used in previous studies, all resulted in a supraphysiological hematocrit levels. When applied to the clinical situation, this could lead to hypertension, seizures, vascular thrombosis and death, possibly related to abruptly increased hematocrit values.[17,19,30] This could be of potential concern in patients at already elevated cardiovascular risk. Therefore, we investigated the effects of a non-erythropoietic EPO dose to avoid elevation of hematocrit, and consequently the changes in rheology and oxygen-binding capacity of the blood. We compared these effects to a high EPO-dose, which effects on cardiac function in post-MI heart failure were already established.[10] Moreover, similar to the high-dose treatment, low-dose treatment resulted in noticeably improved cardiac function, reflected by a significantly enhanced developed LVP, together with improved contractility

and relaxation indices of the LV. Similar to high dose EPO, low-dose EPO induced neovascularization and attenuated the unfavourable switch to slow β-MHC isoforms. However, in contrast to high-dose EPO, LVEDP was not significantly attenuated in the low-dose group compared to the untreated MI. Thus, in spite of increased filling pressures, low-dose EPO improves contractile properties of the non-infarcted part of the myocardium. Although the (non significantly) larger infarct size in the low-dose EPO group could have partially averted the beneficial effects of EPO, neovascularization was also less pronounced in the MI-EPO-low group. This might indicate that part of the beneficial effects are related to an increased hematocrit and consequently increased oxygen delivery. However, the low dose EPO group was treated with a dose 100 times lower than the high dose group. Therefore the slightly less pronounced effects in the MI-EPO-low group might also reflect a reduced magnitude of the non-erythropoietic effects. The anti-apoptotic effects of EPO in the myocardium have been well described in the setting of acute ischemia, yet EPO did not reduce apoptotic cells in our study. Although these results might seem contradictory to previous reports, apoptosis was determined 3 weeks after the last EPO-dose, when EPO-levels are comparable between groups.[14] Since the anti apoptotic effects of EPO are dependent on EPO receptor signalling, possible effects of treatment will have elapsed.

Application of lower doses of EPO was also shown to confer vascular and tissue protection in the kidney.[22] Low-dose darbepoetin treatment in a rat remnant kidney model improved the survival, ameliorated endothelial damage and preserved renal function, without an increase in hematocrit levels. In contrast, Prunier et al. recently reported that a weekly dose of 0.75 μg/kg darbepoetin alfa, initiated 1 week after myocardial ischemia reperfusion injury, was insufficient to improve cardiac function and induce neovascularization.[9] The dose utilized by Prunier et al. was clearly higher than the present study and resulted in significant elevation of hematocrit. The reason for the discrepancy between the study by Prunier et al. and our study is unclear. Possibly, the relatively limited infarct size associated with ischemia reperfusion injury, and the consequently limited decline in cardiac function, might result in more equivocal results. Furthermore, since increased hematocrit levels might have deleterious effects, the balance between the negative effects of hematocrit elevation and the beneficial effects of EPO might become unfavourable.

Another option to circumvent unwanted effects of EPO on hematocrit, could be the use of recently discovered non-erythropoietic derivates of EPO, retaining the tissue protective property, without undesired effect on erythropoiesis.[21] The possibility to separate the erythropoietic and tissue-protective effects could be explained through interaction of EPO with different receptors in bone marrow and in "peripheral" tissues. Two independent studies have demonstrated that these non-erythropoietic EPO's retain their acute cardioprotective potential.[31,32] It is however uncertain whether these new EPO's will also improve cardiac function in CHF. Finally, a very recent study by Schneider et al. revealed that endocardial EPO injections improve the contractile function of hibernating myocardium, without affecting hematocrit levels.[33] One of the mayor shortcomings of the present study is the lack proper evaluation of platelet activation and coagulability. Apart from differences in hematocrit values, the beneficial

effect low-dose EPO might include attenuated EPO-induced activation of platelets and coagulability. Although some reports have described enhanced coagulability in patients treated with EPO, a link between hematopoiesis-stimulating drugs and thrombosis has not been proven. Indeed, Lindenblatt et al. have recently demonstrated that high dose darbepoetin (10 μg/kg/week) did not affect coagulation and platelet aggregration in mice. Since we used an 8 times lower dose of EPO in the present study, similar results are expected, but should be proven in future studies.

Conclusions

In summary, EPO treatment improves cardiac function and induces neovasculari-sation in post-MI heart failure, even in a dose that doses not increase hematocrit. Although time-limited treatment with high-dose EPO may be beneficial and safe during acute ischemic injury, if prolonged therapy is required (heart failure), drug regimens using low-dose EPO may be more suitable to avoid the adverse effects of the treatment.

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Chapter 9

Vascular endothelial growth factor production by cardiomyocytes mediates erythropoietin induced restoration of cardiac function in heart failure

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Submitted

Abstract

Background

Erythropoietin (EPO) stimulates Vascular Endothelial Growth Factor (VEGF) expression in the heart. We aimed to delineate the cardiac cell types and signal transduction pathways responsible for EPO induced cardiac VEGF production and establish whether VEGF is responsible for EPO-induced improvement of cardiac function in heart failure.

Methods and results

EPO increased VEGF expression in cultured neonatal rat cardiomyocytes but not in cultured human umbilical vein endothelial cells. Augmented VEGF production was blocked by the STAT-3 inhibitor pPYLKTK-mts but not by the PI3K inhibitor wortmannin or the ERK-inhibitor PD98059. This was confirmed in a rat aortic sprouting assay where the effects of EPO on sprouting of endothelial cells were modest and VEGF independent. In rats with heart failure after myocardial infarction (MI), EPO treatment (darbepoetin alfa 40μg/kg/3weeks, starting 3 weeks after MI) resulted in as 3-fold increased expression of VEGF in cardiomyocytes and significantly improved cardiac capillary density (37 %, $P<0.01$) and left ventricular function (all $P<0.01$ vs untreated MI). Neutralization of VEGF with two distinct antibodies abrogated the salutary effects of EPO on left ventricular microvascularization and function (P=NS vs MI, $P<0.01$ vs MI-EPO). VEGF neutralization attenuated EPO-induced proliferation of myocardial endothelial cells ($P=0.01$). In addition, neutralization of VEGF reduced myocardial homing of endothelial progenitor cells by 42% in rats with alkaline phosphatase labeled bone marrow cells.

Conclusions

EPO stimulates STAT-3-mediated VEGF production by cardiomyocytes, which in turn mediates EPO induced neovascularization and improvement of cardiac function in heart failure.

Introduction

Although significant morbidity and mortality benefits have been made, chronic heart failure (CHF) remains a prevalent medical condition with a poor prognosis. The development of new therapeutic strategies is therefore of utter importance. [1]

A key pathophysiological feature that contributes to progressive cardiac dysfunction in heart failure is insufficient microvascular adaptation to cardiomyocyte hyperthrophy.[2,3] We and others have extensively shown that treatment with EPO restores microvascular insufficiency, and improves cardiac performance in experimental and clinical heart failure.[4-7] The mechanisms of EPO-induced neovascularization in heart failure are however incompletely understood.

There are several reasons to believe that activation of Vascular Endothelial Growth Factor (VEGF) is involved the EPO-induced cardiac effects. First, EPO increases VEGF expression in various ischemic tissues and cardiac VEGF levels are strongly correlated with new vessel formation.[8-11] Second, EPO only stimulates neovascularization in the heart at sites where VEGF expression is increased.[12] Third, mice that lack an EPO-receptor (EPO-R) in the heart display defective VEGF-expression and dramatically accelerated development of left ventricular (LV)-dysfunction during pressure overload.[9] Therefore, myocardial up regulation of VEGF seems a pivotal process in EPO-induced stimulation of myocardial neovascularization. However, the direct effects of EPO on VEGF expression and its relation to new vessel formation and improvement of cardiac function have not been studied.

We hypothesized that VEGF up regulation in the myocardium is crucial for EPO-derived restoration of cardiac microvascularization and performance. We aimed to identify cell types and signal transduction pathways responsible for EPO-induced augmentation of VEGF in the heart and establish whether augmented VEGF expression is crucial for EPO-induced improvement of cardiac microvascularization and function.

Materials and methods

Effect of EPO on VEGF-transcription in cardiomyocytes and endothelial cells

Neonatal rat cardiomyocytes (NRCMC, from Sprague Dawley rats, Zeist, The Netherlands) and Human Umbilical Vein Endothelial Cells (HUVECs) were isolated and maintained as described previously.[13,14] NRCMC (10^5 cells / well) were incubated without serum for 22 hours and HUVECs cultures at 90% confluence were incubated in RPMI1640 medium with L-glutamin and 2% fetal bovine serum for 18 hours. Because we have previously described that ischemia is required for EPO-induced upregulation of VEGF in the heart, cells were pre-incubated with or without the hypoxia mimetic deferoxamine (100 μM, Sigma Aldrich) for 2 hours. The role of well described EPO-receptor signal transduction pathways was studied

by additional pre-incubation with the PI3-Kinase blocker wortmannin (1 μM, Sigma Aldrich), the MEKK-kinase blocker PD98059 (25 μM, Sigma Aldrich), or the cell permeable STAT3-inhibitor peptide (pPYLKTK-mts, 1 μM, Calbiochem) for 30 minutes. After pre-incubation, EPO (Eprex, Janssen-Cilag, final concentration of 10 IU/mL), was added to the wells and cells were lysated after 30, 60 or 120 minutes with TRIZOL-reagent (Invitrogen) and RNA was isolated according to the suppliers guidelines. After reverse transcription with random primers (Promega), quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) was performed with PCR-master mix containing cyber green (AbGene) or with TAM-labeled primer / probe sets according to the suppliers guidelines (Applied Biosystems). B2M was used as a housekeeping gene in all analysis and data are expressed as fold-difference compared to control. We analyzed at least 8 wells per group and experiments were divided over at least 3 separate experiments with cells from different donors.

Aortic sprouting assay

The aortic ring assay, which is a co-culture of endothelial cells, fibroblasts and vascular smooth muscle cells, was performed using the method of Nicossia and Ottinetti with slight modifications.[15] Briefly, 0.6 mm long aortic rings of the thoracic aorta were embedded in growth factor reduced Matrigel (Becton Dickinson). The aortic rings were then cultured in endothelial cell culture medium (which contains endothelial cell growth factor (ECGF) and heparin)[14] with or without 10 IU/mL EPO or 1 μg/mL goat anti-rat VEGF (AF 564, R&D systems) for 7 days. Maximal sprout length was measured with Image-Pro (Version 4.5.0.29) and is expressed in arbitrary units.

Animals and bone marrow labeling

Male Sprague Dawley rats (270-320 g) were purchased from Harlan (Zeist, the Netherlands). For bone marrow transplantation experiments we used male Fischer F344 rats (200-230 g) purchased from Charles Rivers (France) as recipients and R26-hPAP donor rats (F344 background, ubiquitously expressing human placental alkaline phosphatase, hPAP).[16] Details on transplantation have been described in detail previously.[8] Briefly, whole R26-hPAP bone marrow cells were transfused to Fischer F344 recipients after total body irradiation and left to reconstitute before commencing with the experimental protocol. Animals were fed and housed, according to institutional rules and regulations. The experimental protocol was approved by the Animal Ethical Committee of the University of Groningen.

Experimental protocol in rats

Because VEGF-signaling is crucial for the normal control of cardiac microvascularization in heart failure,[17] we inhibited VEGF signaling by neutralizing antibodies only in the first week after EPO treatment. Myocardial Infarction (MI) was induced by permanent ligation of the left coronary artery.[5] In sham operated rats, the same surgery was performed without closing the suture. Three weeks after MI, rats were randomly assigned to treatment with the long acting EPO analogue darbepoetin alfa (40 μg/kg/3 weeks, Aranesp, Amgen Inc., Thousand Oaks, CA, USA) or vehicle. Rats

were additionally randomized to treatment with goat anti rat-VEGF affinity purified antibody (5 μg/rat aVEGF1; R&D systems, AF564) or the irrelevant control antibody goat anti-mouse-IgG (5 μg/rat, R&D systems, AF007) three times per week during one week immediately following each EPO-administration. To control for potential non-specific effects of antibody cross reactivity, we additionally treated separate groups of rats with mouse anti-humanVEGF165 (aVEGF2, 1 mg/rat). This resulted in 7 treatment groups, 3 treated with irrelevant control antibody (sham, untreated MI, MI-EPO) and 2 with each VEGF neutralizing antibody (MI-EPO + aVEGF1, MI + aVEGF1, MI-EPO + aVEGF2, MI + aVEGF2). To evaluate the temporal characteristics of VEGF-neutralization, blood was drawn from the tail vein at week 3, 4, 6 and 7. After 9 weeks, at sacrifice, hemodynamic measurements were performed with a microtip pressure transducer (Millar Instr. Inc., Houston, Texas, USA) as described previously.[18] Heart rate (HR), left ventricular systolic pressure (LVSP), and left ventricular end-diastolic pressure (LVEDP) were measured. The maximal rates of increase and decrease in LVP (dP/dt_{max} and dP/dt_{min}) and the developed LV-pressure (dLVP) were determined. Next, whole blood was drawn and hearts were rapidly excised and weighed. Myocardial tissue was dissected transversally and processed for immunohistochemistry or snap frozen for molecular analysis.

VEGF-neutralizing capacity of plasma

96-well plates were coated with donkey anti-goat-IgG in PBS overnight. Next, plates were washed 5 times and incubated with plasma samples of 4 rats that were treated with the irrelevant control antibody and 4 rats that received aVEGF1. Recombinant humanVEGF165 (R&D systems) was added to the wells to reach a final concentration of 1000 pg/mL and incubated for 60 minutes. Hereafter, VEGF concentration was determined by ELISA according to the suppliers' guidelines. (R&D systems) Percentage neutralization was determined as VEGF-measured pg/mL / 1000) * 100%. The neutralizing characteristics of aVEGF2 have been described elsewhere.[19]

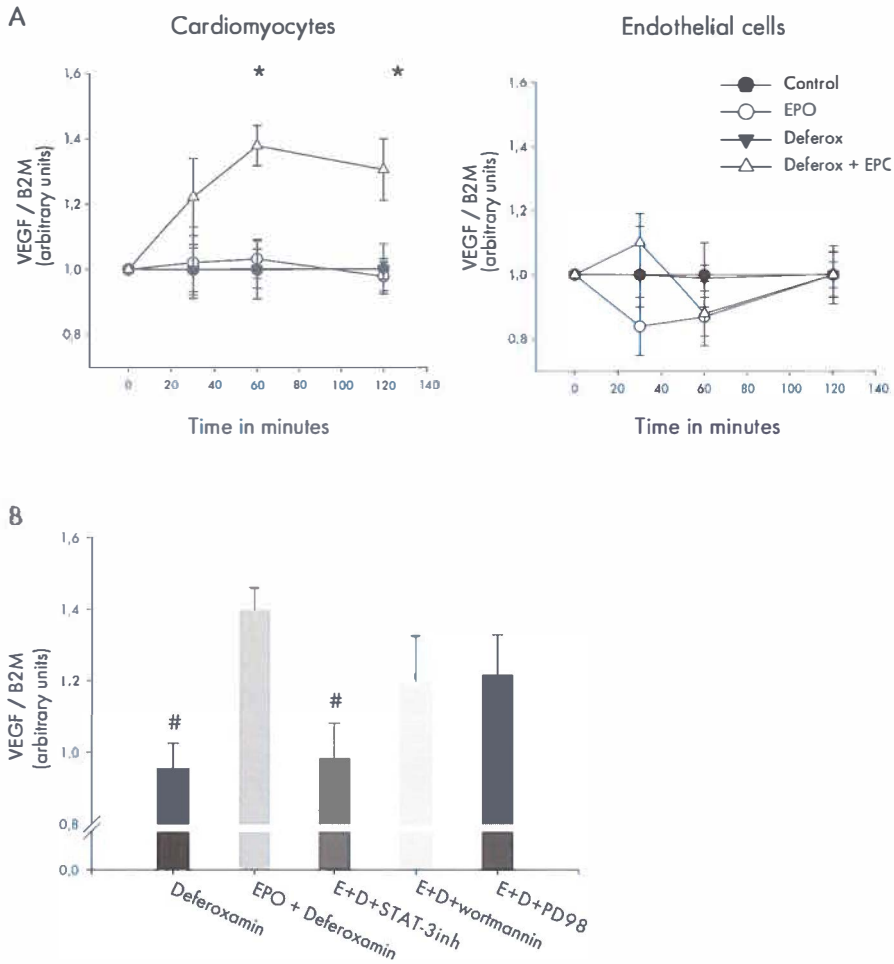
Myocardial VEGF protein content

The expression of VEGF and GAPDH was determined in tissue homogenates of the viable left ventricular free wall (non infarcted area) by standard Western blotting techniques as described previously.[8]

Circulating endothelial progenitor cells

Circulating EPCs were enumerated as previously described.[8] Briefly, mononuclear were seeded onto fibronectin-precoated wells in EndoCult medium (StemCell Technologies). After 6 days, cells were incubated with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine labelled acetylated-LDL (DiL-AcLDL) for 12 hours, fixed with 1% paraformaldehyde and counterstained with fluorescein-labelled isolectin B_4. Cells double positive for DiI AcLDL and lectin staining were considered EPCs and counted in 5 high-power fields, by blinded observers (BDW, WPTR).

Figure 1. Effects of EPO on VEGF gene transcription in cardiomyocytes and endothelial cells.

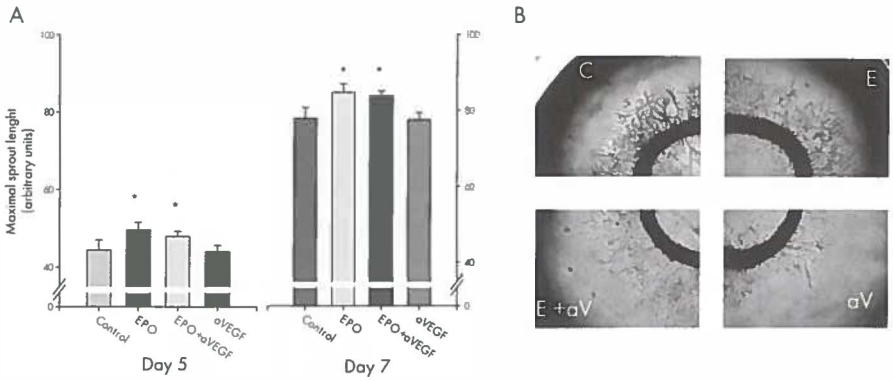


A. Graphic representation of temporal changes in VEGF mRNA expression after EPO-treatment in neonatal rat cardiomyocytes (NRCMC) and endothelial cells (HUVECs) in the presence or absence of the hypoxia inducing agent deferoxamine. **B.** Graphic representation of VEGF mRNA expression in EPO-treated NRCMC with or without specific blockers for EPO-receptor signal transduction pathways. E; EPO, D; deferoxamine, STAT-3inh; STAT-3 inhibiting peptide, PD98; PD98059.

Left ventricular histology

Cardiomyocytes' cross sectional area and the number of cardiomyocytes / mm^2 were determined after Gomoris' silver staining as described previously.[5] Deparaffinized sections were stained with the primary antibodies mouse anti Troponin T (JLT-12, Sigma Aldrich), rabbit anti VEGF (A20, Santa Cruz biotechnology), mouse anti-Proliferating Cell Nuclear Antigen (PCNA, PC10, Cell Signaling Technology) or biotin labeled GSL-

Figure 2. Effects of VEGF inhibition on EPO induced in vitro angiogenesis.



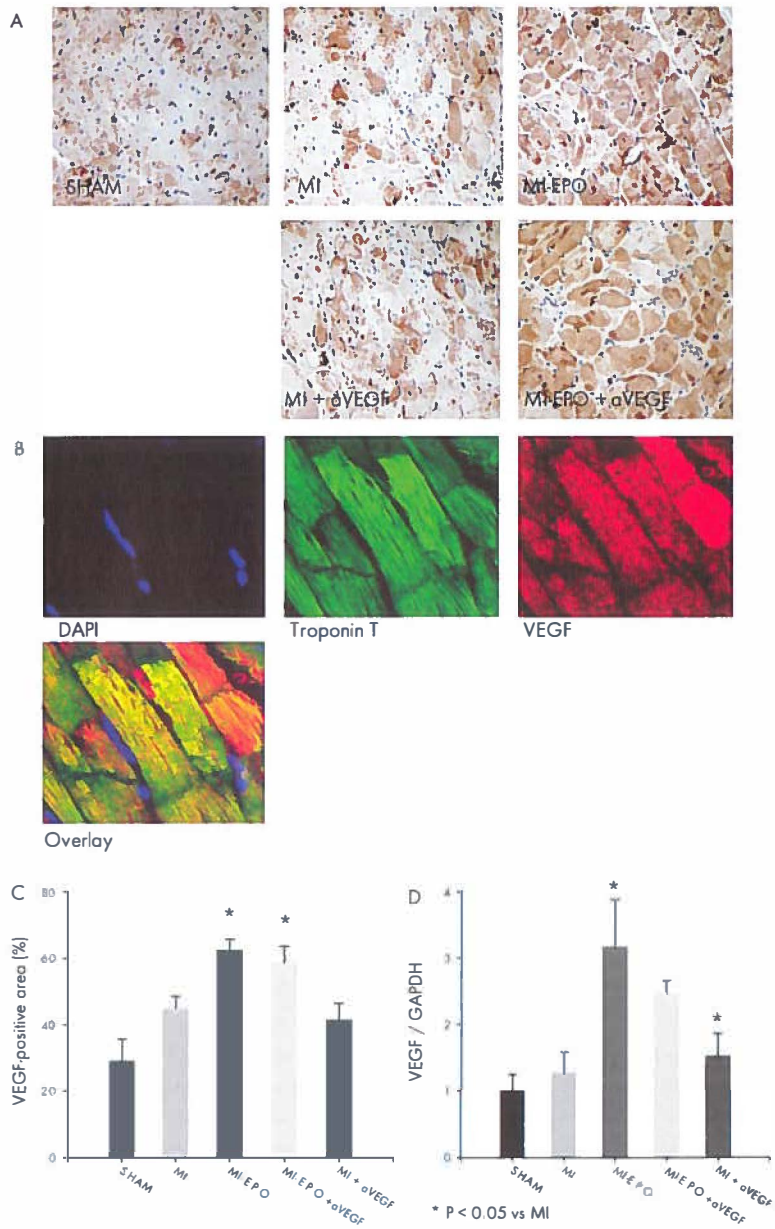
A. Graphic representation of the maximal aortic sprout length after 5 and 7 days in a matrigel aortic implantation assay. **B**. Typical examples of aortic sprouting in the different experimental groups. C: Control; E: EPO; aV: VEGF-neutralising antibody.

Lectin (Sigma Aldrich). For chromogenic detection, Streptavidin-HRP (Santa Cruz) or the Envision kit (Dako-Cytomation) were used and detection was performed with diamynobenzydine (DAB)-staining with mayers hematoxcillin (Sigma) for nuclear counterstaining. For fluorescent detection, streptavidin-FITC (Perkin Elmer), anti-mouse Alexa555 (Invitrogen) or goat anti-Rabbit-HRP followed by the biotynylrhodamine-TSA kit (Perkin Elmer) were used. Cyosections were stained for hPAP (rabbit anti-hPAP, serotec, London, U.K. followed by FITC labelled goat anti-rabbit-IgG) and rat endothelial cell antigen (RECA; mouse anti-rat His52; a kind gift from dr. J.L. Hillebrands, followed by TRITC-labeled goat anti-mouse-IgG). Diamidino-2-phelylindole (DAPI) was used for all fluorescent nuclear staining. Infarct size was determined at the mid-papillary level and capillary density was determined as the number of capillaries per tissue area (mm^2) after GSL-Lectin staining as described in detail previously,[5] with the addition of 1 minute eosin-staining to enhance evaluation of tissue surface area. VEGF-positive area of the myocardium was determined with Image pro (version 4.5) after DAB staining and expressed as percentage of the total area. Cells double positive for lectin and PCNA were considered proliferating endothelial cells, and cells double positive for hPAP and His-52 were considered bone marrow derived endothelial cells. Quantification of double positive cells was performed in 4-5 random high power fields of the non-infarcted left ventricular free wall and were counted independently by blinded observers (BDW, LY).

Statistical methods

Data are expressed a s mean ± SEM. Statistical analysis among groups was performed by ANOVA with the bonferroni post hoc test if distributed normally or with the Kruskall-Wallis test followed by Mann Whitney-U when skewed distributed. All P-values are two-tailed, and a P-value of <0.05 was considered statistically significant. All analyses were performed using SPSS version 15.0 software (SPSS, Chicago, IL, USA).

Figure 3. Effect of EPO on VEGF expression in the myocordium.



A. Typical examples of VEGF-immunohistochemistry of myocardial sections of treatment graups. **B**. Immunofluorescent staining of rat myocardium. Co-localization of VEGF (red) and troponin-T (green) confirms that VEGF is predominantly expressed in cardiomyocytes. **C**. Graphic representation of VEGF immunareactive surface area. **D**. Graphic representation of the VEGF protein expression in the left ventricular free wall.

Results

Effects of EPO on VEGF-transcription in cardiomyocytes and endothelial cells

VEGF mRNA expression was significantly increased by EPO in NRCMC only in the presence of the hypoxia-mimicking agent deferoxamine (P=0.02, figure 1A). In HUVECs, VEGF-expression was unaffected by EPO (P=non-significant (NS), figure 1A). Extended incubation with deferoxamine increased VEGF-expression in NRCMC and HUVECs compared to cultures without deferoxamine, but did not alter the effects of EPO on VEGF-expression (data not shown). The effects of EPO on VEGF expression were blocked by STAT-3 inhibiting peptide, but not by wortmannin or PD98059 (figure 1B), suggesting that the STAT-3 pathway is the operative in this signal.

Role of VEGF in EPO-induced aortic sprouting

To corroborate the previous findings, we studied the effects of EPO in the aortic sprouting assay, which is a co-culture of vascular cells without parenchyma cells such as cardiomyocytes. EPO significantly increased maximal sprout length compared to control cultures, but the increase was less than 10% ($P<0.05$, figure 2). Neutralization of VEGF did not inhibit the effects of EPO on aortic sprouting.

Effect of EPO on VEGF-production in rats with heart failure

In rats with post-MI heart failure, EPO treatment resulted in a 3-fold increased protein expression of VEGF in the viable left ventricular free wall (figure 3D). In addition to increased protein levels, EPO-treatment also increased the VEGF-immunoreactive area, suggesting that EPO additionally increased the number of cells that produced VEGF (figure 3 A+C). Immunofluorescent double staining showed that VEGF expression was especially apparent in cardiomyocytes (figure 3B). VEGF neutralization did not significantly alter VEGF-expression (figure 2).

Effect of VEGF neutralization on EPO-induced improvement of cardiac function

A total of 150 Sprague Dawely rats were used. Peri-operative mortality in MI rats was 42% and 8% of rats were excluded because the infarct size was below 25%, leaving 75 rats for analysis. Characteristics of the study groups are presented in table 1. Immediately following antibody administration, plasma of aVEGF1 treated rats neutralized 78% of VEGF. In the subsequent weeks without antibody administration, values were normalized to the irrelevant control antibody, indicating transient neutralization of VEGF only during EPO treatment. EPO resulted in a significantly increased hematocrit and augmented numbers of circulating EPCs. VEGF neutralization did not affect hematocrit levels or circulating EPC-numbers (Table 1). MI resulted in a significant increase in heart weight and cardiomyocytes cross-sectional area, which was influenced neither by EPO treatment nor by VEGF inhibition (Table 1). Hemodynamic measurements are presented in table 1 and figure 4. Induction of MI resulted in a decreased cardiac function in all MI groups (figure 4).

Table 1. Characteristics of the experimental groups at sacrifice (week 9)

Variable	SHAM + IgG	MI + IgG	MI-EPO + IgG	MI + EPO + aVEGF1	MI + EPO + aVEGF2	MI + aVEGF1	MI + aVEGF2
N	9	9	11	13	10	11	12
Infarct size	-	41 ± 2.3	42 ± 1.8	43 ± 1.5	43 ± 2.0	43 ± 2.1	44 ± 1.8
Bodyweight	396 ± 7	409 ± 16	414 ± 7	406 ± 6	415 ± 9	416 ± 10	402 ± 13
Hematocrit	47 ± 1.2	48 ± 1.2	58 ± 1,5†‡	56 ± 0.9†‡	55 ± 2.6*#	49 ± 1.4	47 ± 0.8
Circ. EPCs	147 ± 15	91 ± 25*	280 ± 62*‡	224 ± 34*‡	-	135 ± 21	-
Heart / body	3.4 ± 0.1	5.2 ± 0.4†	4.9 ± 0.3†	4.7 ± 0.3†	4.4 ± 0.1†	5.0 ± 0.3†	5.4 ± 0.8†
Cardiom-cross.	405 ± 28	771 ± 46†	806 ± 41†	756 ± 24†	806 ± 37†	781 ± 47†	804 ± 28†
Lung / body	4.4 ±0.1	8.1 ± 1.7†	8.0 ± 1.3†	8.1 ± 1.1†	8.4 ±1.1†	10.6 ± 1.2†	7.9 ± 1.0†
Hemodynamics							
Heart rate (bpm)	298 ± 6.5	303 ± 19	317 ± 6	292 ± 8	300 ± 13	293 ± 8	291 ±5
LV-syst (mmHg)	119 ±4	102 ± 2†	112 ± 4†‡	101 ± 2†	105 ± 4†	100 ± 3†	98 ±2†
Aorta-syst (mmHg)	123 ± 3	101 ±3†	112 ± 3#	100 ± 3†	103 ± 3†	96 ± 6†	98 ±2†
Aorta-diast (mmHg)	80 ± 3	71 ± 3	82 ± 2	74 ± 4	73 ± 3	65 ± 6	71 ± 1

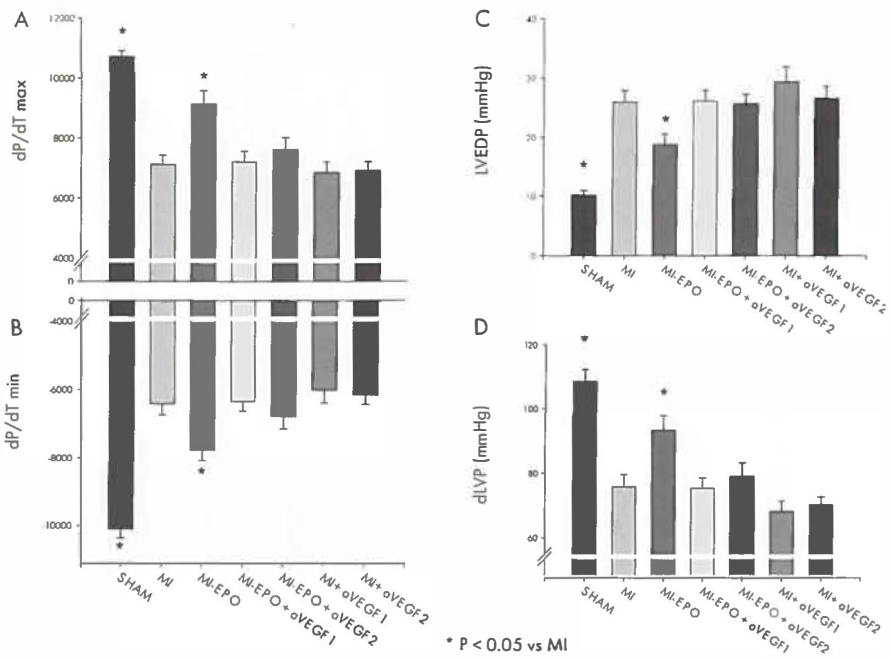
Data are presented as mean ± SEM; N, number of animals; Circ. EPCs, circulating endothelial progenitor cells, Heart / Body; Heart weight / body weight ratio, Cardiom-cross.; cardiomyocytes crossectional area, Lung / Body; Lung weight / body weight ratio, bpm, beats per minute, LV, left ventricle; mmHg, millimeters mercury; syst., systolic; diast, diastolic. * p<0.05; † p<0.01 vs sham, # p<0.05, ‡ p<0.01 vs MI.

EPO-treatment significantly increased left ventricular (LV) contractility (dPdT-max, dLVP) and LV-relaxation (dPdT-min) and decreased LV-filling pressures (LVEDP) compared to the untreated MI (all $P < 0.01$, figure 3). Neutralization of VEGF with both antibodies blocked the salutary effects of EPO on cardiac function parameters (P=NS vs untreated MI, $P < 0.02$ vs MI-EPO, figure 4), while intermittent neutralization itself did not further decrease cardiac function compared to the untreated MI. (P= NS, figure 4). Systolic and diastolic blood pressure decreased after MI and EPO-treatment resulted in a significant increase in systolic blood pressure compared to the MI group ($P<0.05$) although values did not exceed those of shams. The effect of EPO on blood pressure was blocked by VEGF neutralization, while inhibition alone had no effect.

Effect of VEGF neutralization on EPO-induced left ventricular neovascularization

Induction of heart failure significantly reduced cardiac capillary density, (3282 ± 182 vs. 1956 ± 151 capillaries / mm^2, in sham vs MI, $P<0.001$, figure 5). EPO-treatment increased capillary density by 37 % (2688 ± 183 capillaries / mm^2, $P<0.01$ vs MI, $P<0.01$ vs sham, figure 5) and VEGF-inhibition completely blocked the effects of EPO on capillary density (p=NS vs untreated MI, $P<0.01$ vs MI-EPO, figure 5). Intermittent neutralization of VEGF did not result in further reduction of capillary density (P=NS vs untreated MI, figure 5).

Figure 4. Effects of VEGF-neutralization on EPO-induced improvement of left ventricular function.



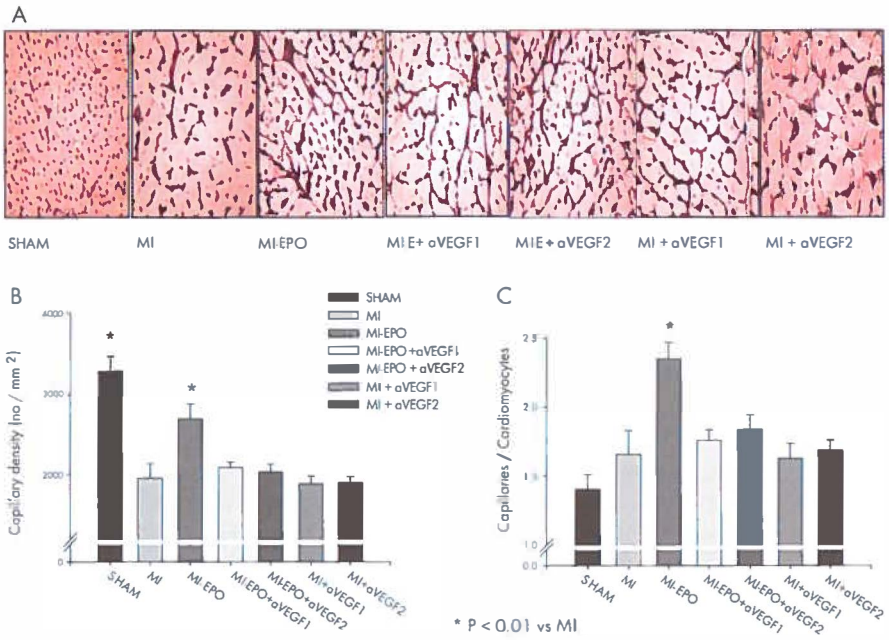
A. Graphic representation of left ventricular contractility (dP/dT max). **B**. Graphic representationof left ventricular relaxation(dP/dT min). **C**. Graphic representation of left ventricular end diastolic pressure (LVEDP). **D**. Graphic representation of the developed left ventricular pressure (dLVP). E, EPO; oVEGF, VEGF neutralising antibodies.

Capillary / cardiomyocyte ratio was slightly but non-significantly increased in all MI groups compared to sham. EPO treatment resulted in a 42% increase in capillary / cardiomyocyte ratio, which was completely abrogated by VEGF inhibition (figure 5).

Effect of VEGF neutralization on EPO-induced proliferation of endothelial cells

The number of proliferating endothelial cells in the viable left ventricular free wall was comparable between sham and MI groups (5.1 ± 0.9 vs 7.6 ± 1.1 cells per field, P=NS, Figure 6 A + B). EPO treatment significantly increased the number of proliferating endothelial cells in the left ventricular free wall (15.7 ± 1.6, P<0.001 vs sham and MI). VEGF inhibition significantly attenuated the stimulatory effect of EPO on endothelial cell proliferation (10.7 ± 0.9 cells / field in MI-EPO + aVEGF, P=0.01 vs MI-EPO) although the number of proliferating cells was still significantly higher than in the sham and MI-group (P<0.05 vs sham and untreated MI).

Figure 5. Effects of VEGF-neutralization on EPO-induced neovascularization.



A. typical examples of capillary density in the experimental groups. **B**. Graphic representation of capillary density expresses as the number of capillaries / mm². **C**. Graphic representation of the capillary / cardiomyocytes ratio. E; EPO.

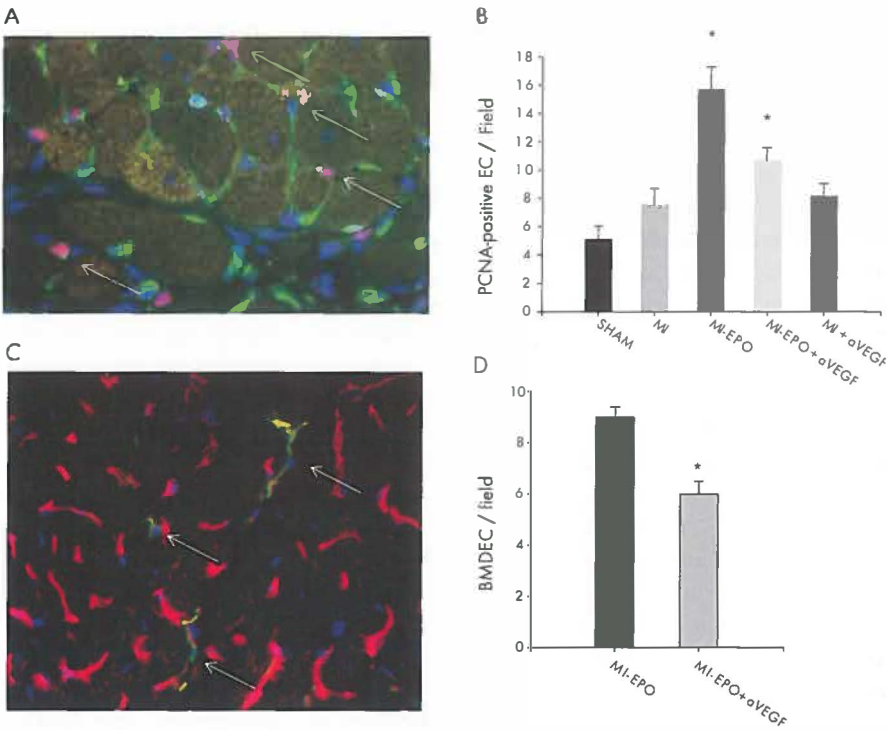
Effect of VEGF neutralization myocardial homing of EPCs

EPO significantly increased circulating EPCs in MI-EPO and MI-EPO + aVEGF groups ($P<0.05$ vs MI). To evaluate the effect of myocardial VEGF upregulation on homing of EPCs we replaced the bone marrow by hPAP-labelled cells, VEGF inhibition reduced the number of bone marrow derived endothelial cells in the left ventricular free wall by 44% (9 ± 0.4 vs 6 ± 0.4 cells /field, $P<0.01$, figure 6 B + C).

Discussion

In the present study we reveal for the first time a crucial role for VEGF in EPO-induced neovascularization and restoration of left ventricular function. In cardiomyocytes, EPO promoted VEGF-transcription through the JAK / STAT-3 signal transduction pathway. In contrast, EPO did not stimulate VEGF-transcription in endothelial cells

Figure 6. Effects of VEGF inhibition on EPO induced myocardial endothelial cell proliferation and vascular incorporation of EPCs.



A. Typical example of immunofluorecent double staining showing PCNA-positive nuclei (red) in capillary endothelial cells (green) highlighted with an arrow. **B**. Graphic representation of the number of proliferating endothelial cells in the left ventricular free wall of the experimental groups. **C**. Representative fluorescent overlay of a myocardial section from an EPO treated animal stained with hPAP (green), His 52 (endothelium, red) and DAPI (nucleus, blue), showing hPAP-positive (ie. bone morrow derived) endothelium, which appearsyellow and is highlighted with arrows. **D**. Graphic representation of the number of bone marrow derived endothelial cells in the left ventricular free wall.

and the direct angiogenic effects of EPO on sprouting of endothelial cells was modest and VEGF independent. In rats with heart failure, EPO stimulated VEGF-production predominantly in cardiomyocytes and neutralization of VEGF completely abrogated the salutary effects of EPO on cardiac function and microvascularization. VEGF was responsible for proliferation of myocardial endothelial cells and homing EPCs to the myocardium. Our study therefore underscores the crucial role for paracrine control of myocardial angiogenesis by cardiomyocytes and proves that the beneficial effects of EPO on cardiac function are truly hematocrit-independent.

EPO restores cardiac function in heart failure by targeting the myocardium

In patients with chronic heart failure, correction of anemia with EPO has been associated with restoration of cardiac function for more than 3 decades.[20, 21] Moreover, in experimental heart failure without anemia, EPO consistently improves cardiac function and microvascularization even in a dose that does not increase hematocrit levels.[22] These observations suggest that EPO mediates these salutary effects by targeting cardiac cells. Our study importantly substantiates this hypothesis. First, we show that EPO-receptor mediated transcription of VEGF by cardiomyocytes conveyed the beneficial effects of EPO on the heart. Second; EPO failed to exert beneficial effect on the heart when VEGF was antagonized, despite a markedly increased hematocrit. Third; although the effect of EPO on the number of circulating EPCs was not affected by VEGF-neutralization, myocardial homing of EPCs and myocardial neovascularization were attenuated, which indicates that EPC-mobilization alone is not sufficient to improve cardiac function in heart failure.

EPO-induced neovascularization is regulated by augmented paracrine secretion of VEGF by cardiomyocytes

Cardiac hypertrophy disproportional to (micro) vascular growth, causes an impaired vascular supply to cardiomyocytes in heart failure.[3] Since VEGF-expression was induced in cardiomyocytes strictly under ischemic conditions, EPO seems to primarily stimulate the normal paracrine angiogenic response of cardiomyocytes to cellular ischemia.[23] Furthermore, EPO-induced VEGF gene transcription was mediated through the JAK / STAT-3 signal transduction pathway. STAT-3 is a crucial transcription factor for growth factor receptor and hypoxia mediated VEGF production.[24] Furthermore, STAT-3 in cardiomyocytes has been identified as an indispensable transcription factor for adaptive angiogenesis to cardiomyocyte hypertrophy.[25] The important role for STAT-3 in EPO-mediated VEGF gene transcription therefore provides additional support for the suggestion that EPO stimulates neovascularization through paracrine mechanisms in cardiomyocytes. The ischemia specific kinetics of EPO-induced VEGF-production in cardiomyocytes might be explained by ischemia-dependent EPO-receptor upregulation.[26] Alternatively, this might indicate that other hypoxia sensing pathways are also operative in this signal. Further studies are required to delineate the mechanisms of the hypoxia-specific nature of EPO.

The finding that the angiogenic effects of EPO are mediated in a paracrine fashion seems to contradict previous studies which showed that EPO markedly stimulates endothelial cell proliferation and vascular tubule formation in numerous *in vitro*-models of angiogenesis.[27] However, although we did observe stimulatory effects of EPO in the aortic sprouting assay, the effects were modest compared to other growth factors. These results confirm recent observations by Asuami et al. whom also demonstrated that the angiogenic effects of EPO on top of VEGF were limited.[9] The angiogenic effects of EPO *in vivo* were however profound and amenable by VEGF neutralization, as evidenced by marked attenuation of PCNA-positive endothelial cells after VEGF-inhibition. Therefore, the effects of EPO on cardiomyocytes seem more important for myocardial angiogenesis that direct stimulation of endothelial cells.

VEGF regulates EPO-induced homing of EPCs to the myocardium

EPO mobilizes EPCs from the bone marrow and we and others have therefore postulated an important role for EPCs in EPO-induced neovascularization. We recently showed that EPO stimulates incorporation of EPCs into the myocardial microvasculature.[8] However, the present study indicates that EPC-mobilization alone does not necessarily augment neovascularization. We previously demonstrated that EPCs incorporated into healthy and diseased tissues, whereas consecutive neovascularization was specifically induced in the presence of VEGF upregulation and ischemia.[12] Moreover, transplantation of normal bone marrow to EPO-receptor null mice does not rescue VEGF-expression and accelerated development of heart failure.[9] One might therefore suggest that EPCs are dispensable for EPO-induced neovascularization. However, attenuated expression of VEGF in these mice was also associated with impaired homing of EPCs.[28] Furthermore, although not affecting circulating EPC-numbers, homing of EPCs in our study was significantly reduced by VEGF neutralization. Thus, in addition to the stimulation of local endothelial proliferation, VEGF seems to serve as an important chemotactic factor for EPO-mobilized EPCs.

Clinical implications

Our study demonstrates that EPO-induced restoration of cardiac function in heart failure is mediated by EPO-receptor signaling in cardiomyocytes. After several promising phase 2 studies,[29-31] a phase 3 clinical trial is currently evaluating the effect of EPO on outcome in CHF patients with anemia. From our data, we propose that trials aimed at restoration of cardiac function in CHF-patients without anemia should circumvent unwanted elevation of hematocrit levels. Ideally, EPO-derivatives that specifically target the EPO-receptor on cardiomyocytes might restore cardiac function without hematopoietic or thrombotic side-effects.[32] Possibly, (post)transcriptional cardiomyocyte-specific modification of the EPO-receptor occurs that might enable us to design cardiac-specific EPO-derivatives. However, we can not completely exclude that the beneficial effects of EPO are in part dependent on direct effects on endothelial cells or EPCs. Hence, efficacy of novel compounds should be compared in an experimental setting before embarking onto clinical trials.

Conclusion

EPO stimulates STAT-3-mediated VEGF production by cardiomyocytes, which in turn mediates EPO induced neovascularization and improvement of cardiac function in heart failure. These findings underscore the non-hematopoietic, pro-angiogenic and salutary effects of EPO in heart failure patients.

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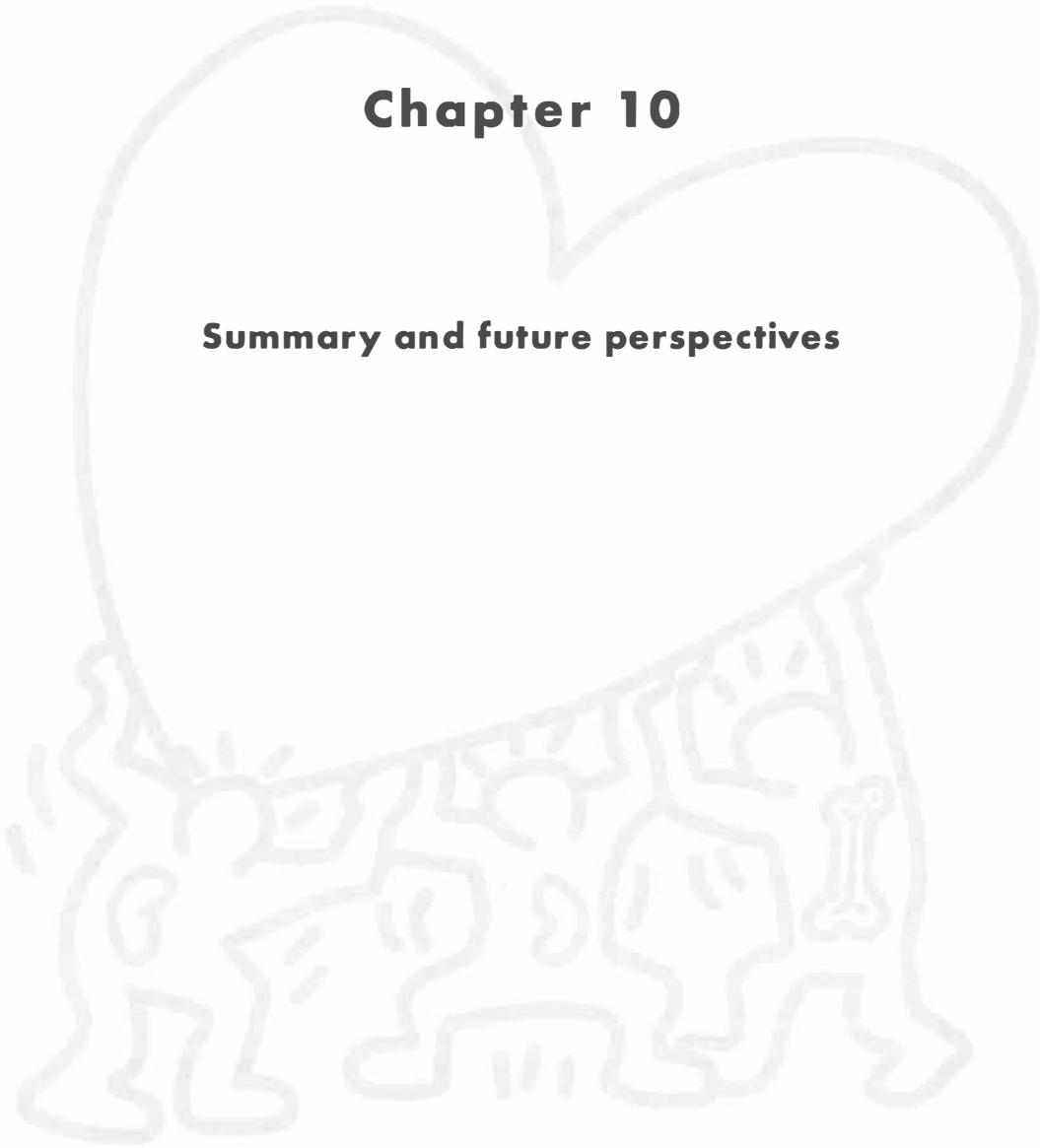
Chapter 10

Summary and future perspectives



Summary

Anemia is common in chronic heart failure (CHF) patients and related to impaired survival. The etiology of anemia in CHF patients is often unknown.[1,2] We hypothesized that dysregulation of erythropoietin (EPO) synthesis by the kidney or an altered sensitivity of the bone marrow to EPO might represent causes specific for CHF. In addition, recombinant human EPO (rhEPO) has been shown to improve cardiac function in heart failure.[3,4] We hypothesized that EPO-induced improvement of cardiac function is mediated through mechanisms that are unrelated to increases in hematocrit levels.[5] In the present thesis we explored EPO as a pathophysiological factor in anemia and the mechanisms of its ancillary beneficial cardiac effects in heart failure.

In the **FIRST PART** of the thesis we explored the role of EPO in anemia of CHF. In **Chapter 1 and 2**, we introduced the clinical significance of anemia in CHF-patients and reviewed the present knowledge of the etiology. In addition, we introduced the extra-hematopoietic EPO-receptor system and discussed the present evidence for a therapeutic potential for rhEPO in cardiovascular disease. In **Chapter 3**, we evaluated whether anemia in CHF patients is caused by renal failure. We hypothesized that impaired renal perfusion in heart failure will compromise EPO-synthesis in the kidney. Alternatively, renal hypoperfusion might cause fluid retention and consequently hemodilution, which can cause anemia as well. Using a novel radionucleotide technique which allows simultaneous evaluation of renal perfusion and extracellular volume, we demonstrated that anemic CHF patients displayed significantly impaired renal function and perfusion compared to non-anemic subjects. Moreover, although EPO levels were slightly higher in anemic CHF patients, the levels were insufficient when corrected for the prevailing hemoglobin (Hb) levels. In addition to blunted EPO-production, anemic CHF patients displayed significantly higher extracellular volumes. Lower renal perfusion, blunted EPO production and higher extracellular volume were all independent predictors of anemia, thereby showing that renal compromise in CHF contributes to the occurrence of anemia through multiple pathways. It additionally signifies the multifactorial etiology of anemia in CHF.

Despite the fact that the EPO-response is blunted in anemic CHF patients, CHF patients generally display relatively elevated EPO levels. This could indicate that the bone marrow of CHF patients is relatively resistant to EPO and would therefore require higher levels to maintain adequate erythropoiesis. In **Chapter 4**, we evaluated the EPO response of hematopoietic progenitor cells of CHF patients. In a well established *in vitro* erythropoiesis assay, $CD34^+$ hematopoietic progenitors isolated from the bone marrow of CHF patients displayed a 3-fold lower number of Burst Forming Units-Erythroid (BFU-E) colonies compared to matched controls with normal cardiac function. Impaired erythropoiesis *in vitro* was accompanied by a relative depletion of the erythroid lineage and markedly increased apoptosis in the bone marrow. Elevated levels of NT-proBNP independently predicted impaired BFU-E formation, indicating that the extent of erythropoiesis-impairment is related to the severity of heart failure.

In contrast to our expectations, EPO-receptor expression was comparable in CHF-patients and controls, suggesting that the sensitivity of erythroid cells to EPO is equivalent. Moreover, BFU-E formation was equally impaired in anemic and non-anemic CHF patients. Therefore, it seems that CHF causes general bone marrow dysfunction which translates into impaired erythropoiesis and might in part explain the high incidence of anemia in CHF. In **Chapter 5**, we postulated a model that explains how heart failure results in changes in the kidney and the bone marrow that eventually culminate into anemia. The model explains why anemia is an intrinsic component of the CHF-syndrome. We also reviewed treatment options, including meticulous control of fluid retention and recombinant human EPO. In addition we reviewed current experimental evidence that EPO has important ancillary effects in heart failure through the induction of myocardial neovascularisation.

In the **SECOND PART** of the thesis we aimed to evaluate the mechanisms through which EPO improves cardiac function in CHF. For this purpose we employed a rat model of chronic post-myocardial infarction (MI) heart failure.[6] In order to study the effects of EPO in a chronically remodelled heart, we initiated treatment 3 weeks after MI, when infarct healing had completed and remodelling of the remaining viable myocardium had occurred. In **Chapter 6**, we evaluated whether bone marrow derived endothelial progenitor cells (EPC)[7] contribute to EPO-induced neovascularization and restoration of cardiac function. By replacing the bone marrow of rats with labelled cells, we showed that EPO-induced restoration of cardiac function and neovascularization is associated with increased mobilization, myocardial homing and vascular incorporation of EPCs. Approximately 30% of the new endothelium comprised of bone marrow derived cells. In addition, Vascular Endothelial Growth Factor (VEGF) protein expression in the myocardium was more than 4-fold increased by EPO and correlated with new vessel formation. VEGF was not primarily expressed by EPCs, suggesting an additional EPC-independent mechanism. The effects of EPO on microvascularization have only been described in the presence of ischemia and might in part be dependent on its presence. In **Chapter 7**, we therefore evaluated whether the effects of EPO on cardiac microvascularization and function only occur in ischemic tissues or represent a general phenomenon. We compared the effects of EPO in rats with ischemic heart failure or with a normal cardiac function. EPO generally stimulated mobilization of EPCs from the bone marrow, which indiscriminately incorporated in the endothelium of ischemic and non-ischemic tissues. Incorporation of EPCs was associated with a significant improvement of endothelial function, independent of ischemia. However, additional EPCs specifically homed to the ischemic MI-borderzone and EPO stimulated neovascularization and improved cardiac function only in ischemic hearts. This was possibly driven by augmented VEGF and EPO-receptor expression. These results suggest that EPO regulates normal EPC- mediated endothelial turnover, but improves cardiac microvascularization and function only in the presence of ischemia.

The experiments described above were all performed with a high dose of EPO, which increases hematocrit to supra-physiological levels. The elevated hematocrit levels

might in part explain the salutary effects, but might also significantly hinder its clinical application.[8, 9] We aimed to evaluate whether EPO can also improve cardiac function in CHF with a dose that does not increase hematocrit levels. In **Chapter 8**, we therefore compared the effects of a regular EPO-dose with a 100-fold lower dose. Low dose EPO did not raise hematocrit levels, but still significantly improved cardiac function. The functional improvement was associated with induction of neovascularization and attenuated expression of slow β-MHC-isoforms. Most of the effects were however slightly less pronounced in the low-dose group. This might indicate that the beneficial effects of EPO in heart failure are partially dependent on an increased hematocrit. Alternatively, they reflect the dose dependent nature of the non-hematocrit related effects of EPO on the heart, which are completely distinct from erythropoiesis.

In the previous chapters we showed that the effect of EPO on EPCs is a relatively generalized phenomenon, while EPO-induces VEGF-expression strictly under ischemic conditions and specifically at the site of neovascularization. Therefore, cardiac VEGF-expression might be crucial for EPO-induced neovascularization. In **Chapter 9** we evaluated the cellular mechanisms of EPO-induced VEGF-upregulation and its necessity for the restoration of cardiac function and neovascularization. *In vitro*, EPO promoted VEGF-transcription through the JAK / STAT-3 signal transduction pathway in cardiomyocytes, but not in endothelial cells. Moreover, in rats with heart failure, EPO-treatment resulted in a 4-fold increase in VEGF expression, which was also predominantly observed in cardiomyocytes. Antagonization of VEGF with neutralizing antibodies abrogated the salutary effects of EPO on cardiac performance and microvascularization. VEGF-neutralization blocked EPO-induced proliferation of endothelial cells and myocardial homing of EPCs. Thus, EPO mediates its effects in heart failure at least partly through stimulation of VEGF-production in cardiomyocytes, which in turn stimulates angiogenesis in a paracrine fashion. It further proves that the beneficial effects of EPO on the failing heart are dependent on EPO-receptor signaling in cardiomyocytes and are truly independent of an increased hematocrit.

Future perspectives

Etiology of anemia in CHF

In the **FIRST PART** of the thesis we demonstrate that anemia in CHF patients is directly related to the extent of cardiac dysfunction.[10] Therefore, (partial) restoration of cardiac dysfunction should restore anemia. Moreover, increased levels of EPO are required in CHF due to bone marrow dysfunction, while EPO production in the kidney is impaired. Production of EPO is thus attenuated in the setting of increased demands, suggesting that correction of anemia with rhEPO is feasible.[10, 11] Moreover, since the second part of the thesis shows that EPO also improves cardiac function through direct stimulation of the heart, rhEPO might not only restore hemoglobin levels but might also prevent the recurrence of anemia. A phase 3 clinical trial is currently evaluating the effect of EPO on outcome in CHF patients with anemia and

will hopefully provide more insight.[12] Nevertheless, bone marrow dysfunction in CHF patients has been demonstrated in multiple hematopoietic lineages and might therefore originate in early stem or progenitor cells.[13-15] Consequently, stimulation of erythropoiesis with rhEPO might correct anemia, but could also accelerate the progression of bone marrow dysfunction by inducing replicative exhaustion in undifferentiated stem or progenitor cells.[16, 17] In addition to anemia, bone marrow dysfunction might have consequences for the cellular immune response and stem cell mediated cardiovascular repair. Future studies are required to delineate the type, extent and the mechanisms of bone marrow dysfunction in CHF both at a cellular and molecular level. Moreover, research on the interplay between cardiac performance, renal perfusion and bone marrow function might reveal a common pathway with therapeutic opportunities.

The present thesis also demonstrates that hemodilution contributes to anemia in CHF, suggesting that meticulous control of fluid retention might be a feasible treatment as well.[18, 19] Although interventional studies are lacking, high dose diuretics or intensive sodium and fluid restrictions might provide a simple but effective treatment for anemia in certain patients. Finally, the multitude of factors that have so far been associated with anemia in CHF indicates that we should not neglect other treatable causes of anemia. For instance, recent studies have shown that intravenous iron might be an effective treatment for anemia caused by iron deficiency in CHF patients.[20, 21] Moreover, the fact that most cardiologists dont perform a proper evaluation of anemia in CHF patients suggests that referring each anemic CHF patients for a proper workup will improve morbidity as well.[22] Finally, it is uncertain whether correction of anemia will improve survival in CHF patients, since anemia might merely be a marker for the severity of cardiac dysfunction.

EPO induced improvement of cardiac function in heart failure

In the SECOND PART of this thesis we demonstrated that EPO-induced improvement of cardiac function in CHF is mediated through neovascularization and is truly independent of an increased hematocrit. Moreover, this thesis demonstrated that although EPO stimulates mobilization and myocardial incorporation of EPCs, it is the presence of myocardial ischemia and the stimulation of myocardial VEGF production which determines neovascularization and subsequent restoration of cardiac function. Therefore, trials with EPO aimed at restoration of cardiac function in CHF-patients should circumvent unwanted elevation of hematocrit.[3, 23] Several approaches might be feasible for this purpose. First, a non-erythropoietic EPO dose as described in chapter 8 might be effective, although the low dose of EPO that is required to circumvent erythropoiesis might have limited cardiac effects as well.[24] Second, since EPO induces neovascularization through direct effects on the heart, myocardial application of EPO might be beneficial without inducing erythropoiesis. Indeed, several studies have shown that intra-myocardial injections of EPO or myocardial implantation of EPO-releasing hydrogels improves cardiac function without increasing hematocrit.[25-27] Third, several EPO analogues have been designed that specifically ligate the EPO-receptor in peripheral tissues but not in the bone marrow, thereby allowing systemic

application of high doses of EPO without increasing hematocrit.[28-30] Since VEGF-secretion by cardiomyocytes is pivotal for the salutary effects of EPO in heart failure, design of cardiomyocyte specific EPO-derivatives might be feasible as well. Possibly, (post)transcriptional cardiomyocyte-specific modification of the EPO-receptor occurs that will enable us to design such cardiac-specific EPO-derivatives. However, we can not completely exclude that the beneficial effects of EPO are in part dependent on endothelial cells or EPCs, since we and others also found direct effects of EPO on these cells.[31] Future studies with cardiomyocyte-specific knock-out of the EPO-receptor might confirm the pivotal role of cardiomyocytes. However, despite accumulating experimental evidence, it remains uncertain whether EPO also improves cardiac function in humans. First proof for such ancillary beneficial effects of EPO might emerge from current trials in anemic heart failure patients or patients with an acute myocardial infarction (NCT00449488 and NCT00378352). In the future, EPO might evolve into a designated heart failure drug with erythropoietic side effects.

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Popular summary in Dutch
(Nederlandse samenvatting)

Samenvatting

Hartfalen is het gemeenschappelijke eindstadium van vrijwel alle hartziekten en vormt de belangrijkste doodsoorzaak in de westerse wereld. De behandeling van hartfalen is voornamelijk gericht op het hart. Echter, wanneer het hart faalt, heeft dit ook gevolgen voor het functioneren van andere organen zoals de nieren, longen, lever en de hersenen. Dit multipele orgaanfalen bepaald in belangrijke mate de prognose van hartfalenpatiënten en vormt daarom mogelijk een aangrijpingspunt voor behandeling. Anemie komt vaak voor bij patiënten met hartfalen en is een onafhankelijke voorspeller van morbiditeit en mortaliteit. Anemie kan daarom ook worden beschouwd als een uiting van multipel orgaanfalen. Hoewel de nadelige gevolgen van anemie voor hartfalenpatiënten al in 1937 zijn beschreven, is de etiologie nog niet opgehelderd waardoor gerichte behandeling ontbreekt.

Erythropoiëtine (EPO) wordt gemaakt in de nier en stimuleert vervolgens de aanmaak van rode bloedcellen in het beenmerg. Omdat de doorbloeding van de nier verstoord raakt bij hartfalen, zou dit de productie van EPO kunnen verstoren. Daarnaast veroorzaakt verminderde nierdoorbloeding vochtretentie, wat kan leiden tot hemodilutie anemie. Daarnaast bestaan er aanwijzingen dat het beenmerg van patiënten met hartfalen minder gevoelig is voor EPO. In **Deel 1** van dit proefschrift onderzochten we de rol van erythropoiëtine (EPO) in de pathofysiologie van anemie bij patiënten met hartfalen. In **Hoofdstuk 1 en 2** wordt de rol van EPO in de aanmaak van rode bloedcellen en de mogelijke rol die EPO speelt bij de pathofysiologie van anemie bij patiënten met hartfalen beschreven. In **Hoofdstuk 3** onderzochten we wat het effect van verminderde nierdoorbloeding is op het ontstaan van anemie. In een groep van 97 hartfalenpatiënten hebben we gelijktijdig de nierdoorbloeding, de EPO-productie en het extracellulaire volume (ECV) gemeten. Hieruit bleek dat patiënten met anemie aanmerkelijk slechtere nierdoorbloeding hadden. Dit leidde vervolgens tot een verminderde EPO-productie door de nier en een verhoging van het extracellulaire volume. Multivariate analyse toonde verder aan dat verminderde nierdoorbloeding, verstoorde EPO productie en een verhoogd extracellulair volume allen onafhankelijke voorspellers waren voor anemie. Verminderde nierdoorbloeding veroorzaakt dus anemie door de productie van EPO te verminderen en het bloed te verdunnen. In **Hoofdstuk 4** onderzochten we of een verminderde beenmergfunctie bijdraagt aan het ontstaan van anemie. Om dit te onderzoeken vergeleken we het vermogen van het beenmerg om rode bloedcellen te vormen tussen patiënten met hartfalen en vergelijkbare proefpersonen met een goede hartfunctie. Het beenmerg van hartfalenpatiënten maakte 3 keer minder rode bloedcellen dan het beenmerg van controle patienten. In tegenstelling tot wat we aanvankelijk verwacht hadden, was er geen verschil in beenmergfunctie tussen anemische en niet anemische hartfalenpatiënten. Hoewel de verstoorde beenmergfunctie het ontstaan van anemie niet volledig kan verklaren, maakt het hartfalenpatiënten waarschijnlijk wel gevoeliger voor anemie. **Hoofdstuk 5** beschrijft een hypothetisch model waarin de relatie tussen verminderde hartfunctie en het ontstaan van anemie wordt verklaard uit de bovenstaande bevindingen.

Naast bekende effecten op de erythropoiëse, is recent ontdekt dat EPO de hartfunctie verbetert bij proefdieren en patiënten met hartfalen. Het is echter nog onduidelijk of de hartfunctieverbetering kan worden toegeschreven aan directe stimulatie van de EPO-receptor in het hart, aan stimulatie van stamcellen uit het beenmerg of toch het gevolg is van een verhoging van het hematocriet. In **Deel 2** onderzochten we het mechanisme van deze cardiale effecten van EPO in ratten met hartfalen. In **Hoofdstuk 6** werd de rol van de zogenoemde endotheel progenitor cellen (EPCs) onderzocht. Deze uit beenmergafkomstige stamcellen migreren naar ischemische weefsels om vervolgens bij te dragen aan de vaatnieuwvorming. Door het beenmerg van ratten te vervangen door gelabelde cellen konden we de bijdrage van de uit beenmerg afkomstige cellen onderzoeken. Hierbij zagen we dat EPO zowel het vrijkomen van EPCs uit het beenmerg als ook de inbouw van deze EPCs in nieuwe bloedvaten stimuleerde. Deze EPCs waren echter verantwoordelijk voor slechts 30 % van de nieuwe vaten. Dit suggereert dat aanvullende mechanismen ook een rol spelen. Zo ontdekten we tevens dat EPO de productie van de vasculaire groeifactor VEGF stimuleerde. In **Hoofdstuk 7** werd onderzocht of de stimulerende effecten van EPO op de vaatnieuwvorming uitsluitend optreden in een ischemisch hart of dat ze ook optreden in gezonde situaties. Daarom vergeleken we de effecten van EPO tussen ratten met ischemisch hartfalen en gezonde ratten. Hieruit bleek dat EPO het vrijkomen van EPCs uit het beenmerg en de inbouw van EPCs in de vaten stimuleerde in zieke en gezonde organen, geassocieerd met een verbetering van de endotheelfunctie. VEGF productie, vaatnieuwvorming en hartfunctieverbetering traden echter uitsluitend op in het falende hart. Het lijkt er dus op dat EPO de inbouw van EPCs algemeen stimuleert terwijl de eigenlijke vaatnieuwvorming alleen optreed in het beschadigde hart waar VEGF vrijkomt.

De onderzoeken die hierboven worden beschreven, werden steeds uitgevoerd met een hoge dosering EPO. Deze hoge dosis stimuleerde niet alleen de hartfunctie maar ook de aanmaak van rode bloedcellen. De verhoging van het hematocriet dat hierdoor ontstaat, zou enerzijds de hartfunctieverbetering op zichzelf kunnen verklaren, maar tevens voor bijwerkingen kunnen zorgen in patiënten met een normaal hematocriet. In Hoofdstuk 8 onderzochten we daarom of EPO de hartfunctie ook kan verbeteren met een dosering die te laag is om het hematocriet te laten stijgen. In ratten met hartfalen vergeleken we de reguliere dosis EPO met een 100 x lagere dosis die geen effect had op het hematocriet. Zowel de hoge als de lage dosering EPO veroorzaakte vaatnieuwvorming en verbeterden de hartfunctie. De effecten van de lage dosering waren echter wel minder uitgesproken. Met dit hoofdstuk werd dus aangetoond dat de gunstige effecten van EPO op het hart onafhankelijk zijn van hematocrietstijging en mogelijk zouden kunnen worden toegepast bij niet-anemische hartfalenpatiënten.

In de hoofdstukken die hierboven zijn beschreven zagen we dat het effect van EPO op EPCs gegeneraliseerd is, terwijl vaatnieuwvorming uitsluitend optreedt op plaatsen waar EPO de VEGF-productie stimuleert. VEGF-stimulatie zou daarom de gunstige effecten van EPO op het hart kunnen verklaren. In Hoofdstuk 9 werd daarom onderzocht of de toegenomen VEGF-productie in het hart cruciaal is voor de gunstige effecten van EPO bij hartfalen. Zowel in celkweek als in het falende hart stimuleerde

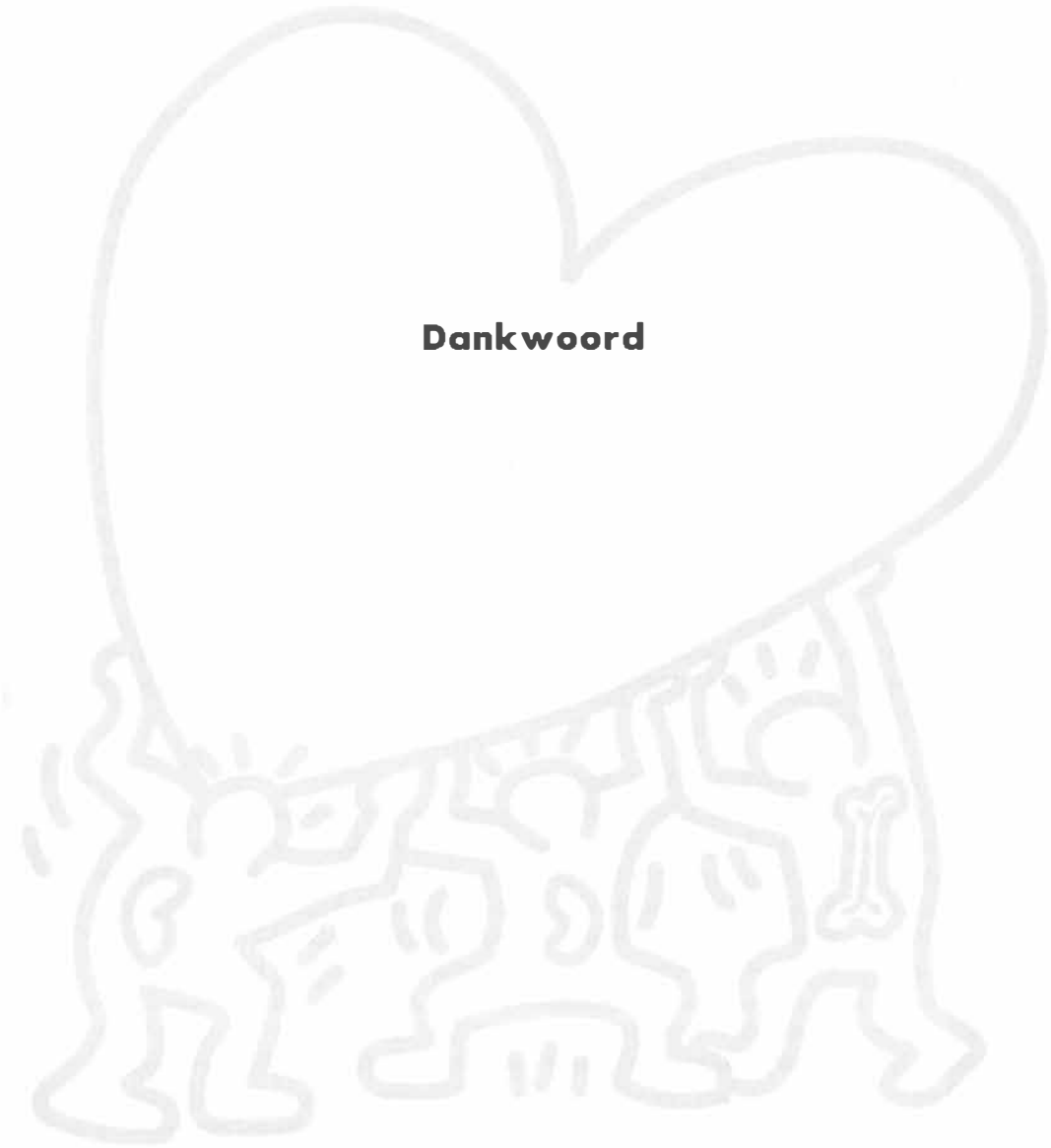
EPO de productie van VEGF in cardiomyocyten, maar niet in endotheelcellen. Wanneer we in ratten met hartfalen tijdens EPO behandeling gelijktijdig VEGF neutraliseerden, was het effect van EPO op zowel de vaatnieuwvorming als de hartfunctie verdwenen. Neutralisatie van VEGF verminderde het aantal delende endotheelcellen en de inbouw van EPCs in het hart. De gunstige effecten van EPO op de hartfunctie kunnen dus zeker ten dele kunnen worden toegeschreven aan de verhoogde VEGF-productie door cardiomyocyten. Naast de cruciale rol voor VEGF tonen we hiermee ook aan dat de directe stimulatie van de EPO-receptor in het hart belangrijker is dan de mobilisatie van EPCs.

Toekomstperspectieven

Uit het eerste deel van het proefschrift blijkt dat anemie bij hartfalenpatiënten gerelateerd is aan de ernst van de hartfunctievermindering. Verbetering van de hartfunctie zou daarom de beste behandeling zijn voor anemie bij hartfalen. Helaas is dit bij veel patiënten niet mogelijk. Verder laten we zien dat de productie van EPO in de nier verstoord raakt terwijl het beenmerg juist meer EPO nodig heeft. Toediening van EPO lijkt daarom een logische behandeling. Uit het tweede deel van dit proefschrift blijkt tevens dat EPO de hartfunctie kan verbeteren bij hartfalen. Hierdoor wordt mogelijk niet alleen de anemie behandeld, maar wordt ook de oorzaak van de anemie (deels) weggenomen. Of EPO ook daadwerkelijk effectief is moet blijken uit aanvullend onderzoek. In dit proefschrift wordt tevens aangetoond dat hemodilutie een rol speelt bij anemie. Dit zou kunnen worden behandeld met stringente vocht en zoutbeperking of diuretica. Verder moeten we er voor waken dat iedere patiënt een goede evaluatie krijgt van de oorzaak van anemie, aangezien er voor veel bekende oorzaken al een behandeling bestaat.

Het tweede deel van dit proefschrift toont aan dat EPO naast effecten op de bloedcelaanmaak ook de hartfunctie bij hartfalen kan verbeteren via directe stimulatie van het hart. Wanneer we de effectiviteit van EPO zouden willen onderzoeken bij mensen zullen we idealiter een methode moeten gebruiken waarmee we optimale EPO concentraties in het hart bereiken zonder dat het hematocriet hierbij stijgt. Hiervoor zijn verschillende mogelijkheden. Ten eerste kunnen er lage doseringen EPO worden toegediend zoals in hoofdstuk 8. Het gevaar bestaat echter dat lage doseringen ook minder werkzaam zijn. Een andere mogelijkheid betreft EPO via injecties direct in het hart te brengen. Als laatste kan gedacht worden aan aanpassingen van het EPO molecuul waardoor het alleen op de EPO-receptor in het hart aangrijpt en zo het beenmerg niet meer stimuleert. Desalniettemin is tot op heden nog niet onomstotelijk bewezen dat de hartfunctie van hartfalenpatiënten ook daadwerkelijk kan worden verbeterd met EPO. Aanwijzingen hiervoor volgen mogelijk uit de onderzoeken met EPO in anemische hartfalenpatiënten en patiënten met een acuut hartinfarct. In de toekomst zou EPO kunnen evolueren tot een specifiek hartfalen medicijn met als hinderlijke bijwerking het stimuleren van de rode bloedcel productie.

Dankwoord



Dankwoord

Aan het tot stand komen van dit proefschrift hebben velen bijgedragen, mijn dank daarvoor. Een aantal van hen wil ik graag met naam noemen.

Mijn promotores prof. dr. D.J. van Veldhuisen en prof. dr. W.H. van Gilst.

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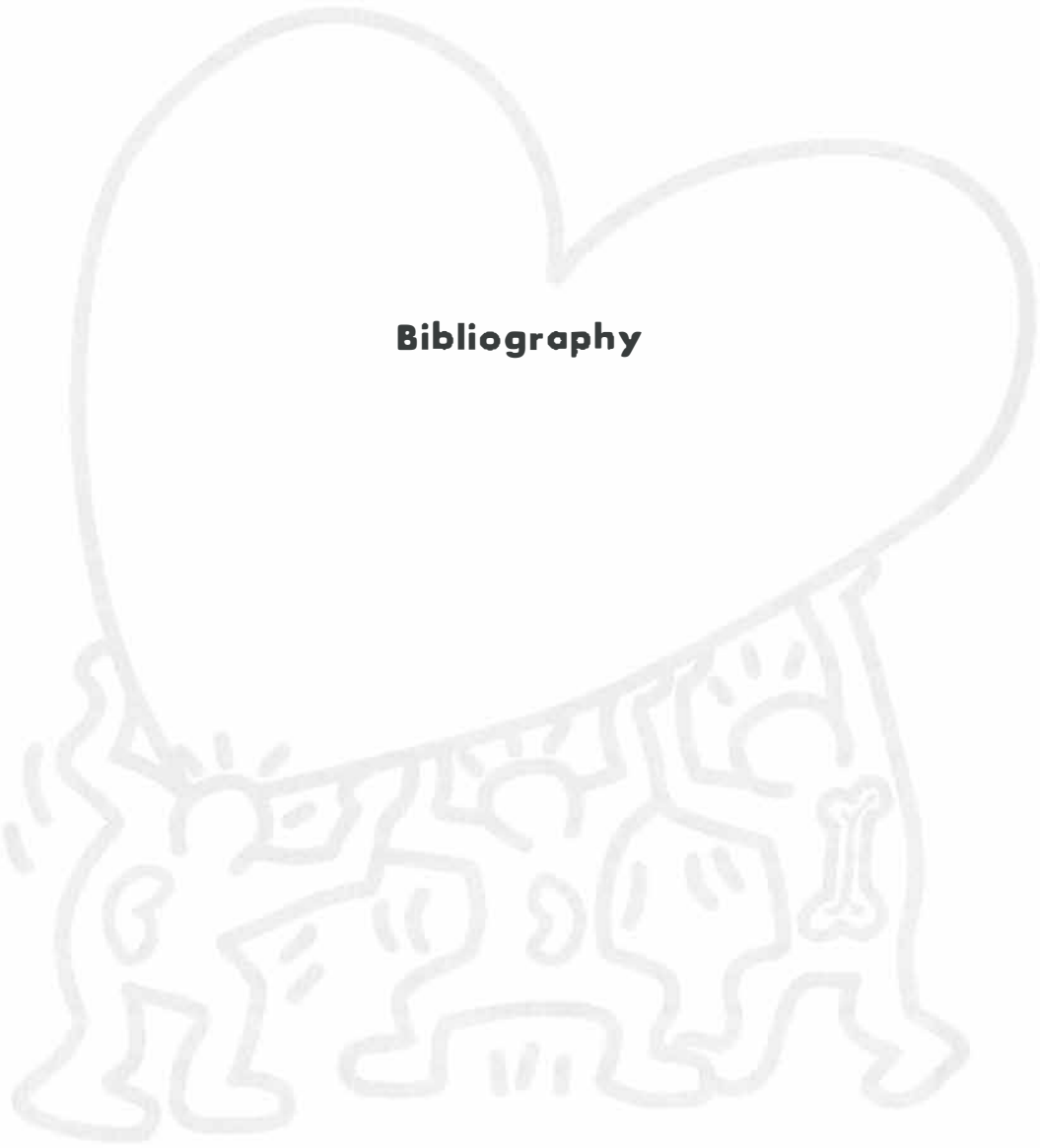

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